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(54) Title: COMPOSITION COMPRISING PLANT GROWTH PROMOTING RHIZOBACTERIA

(57) Abstract

Compositions for enhancing grain yield and protein yield of a legume grown under environmental conditions that inhibit or delay nodulation thereof are provided. The compositions comprise a PGPR strain. In a particular embodiment the PGPR strain is of the genus *Serratia*. Moreover, methods for enhancing grain yield and protein yield of a legume grown under environmental conditions that inhibit or delay nodulation thereof are provided. The methods comprise an addition of an agriculturally effective amount of a PGPR strain in the vicinity of the seed or root of the legume.

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TITLE OF THE INVENTION

COMPOSITION COMPRISING PLANT GROWTH PROMOTING RHIZOBACTERIA

FIELD OF THE INVENTION

The present invention relates to compositions comprising plant growth promoting rhizobacteria (PGPR) and methods therefor for enhancing the formation and development of root nodules in legumes, so as to enable an increase in the growth and yield thereof under conditions that inhibit or delay nodulation. More particularly, the invention relates to compositions comprising PGPR and methods therefor for increasing grain yield and protein yield of soybean grown under environmental conditions that inhibit or delay nodulation, such as suboptimal root zone temperatures (RZTs).

BACKGROUND OF THE INVENTION

The knowledge that elements in the soil influence root nodulation has long been recognized. Indeed, the Romans transferred soil from successful legume fields to unsuccessful ones in order to improve the quality of the latter.

It has since then been demonstrated that one important soil element responsible for nodulation is soil bacteria. The family *Rhizobiaceae* consists of a heterogeneous group of gram-negative, aerobic, non-spore-forming rods that can invade and induce a highly differentiated structure, the nodule (on the roots, and in some instances, stems of leguminous plants), within which atmospheric nitrogen is reduced to

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ammonia by the bacteria. The family *Rhizobiaceae* contains three genera, *Rhizobium, Bradyrhizobium*, and *Azorhizobium*. The host plant is most often of the family *Leguminosae*. The slow-growing nodulation bacteria which have specific associations with soybean are referred to as *Bradyrhizobium*. Currently, *Bradyrhizobium* has only one named species *B. japonicum*, with others lumped together in a miscellaneous group (Barbour et al., 1992); these latter strains are referred to as *B.* sp., followed by the plant species they infect in parenthesis. Some soybean plants can also nodulate with the fast growing *Rhizobium fredii* (Sprent et al., 1990). *Rhizobium* species, sometimes designated "fast-growing" rhizobia, include among others *R. meliloti* which infects alfalfa.

The element N is essential to all living organisms because it is a component of many biologically important molecules. The most important of these include nucleic acids, amino acids and therefore proteins, and porphyrins, which occur in large amounts in all living cells. To be able to multiply and grow, or just survive, organisms require a source of N. The ability to reduce atmospheric dinitrogen is limited to prokaryotes. Legumes and a few other plant species have the ability to fix atmospheric N through symbiotic relationships. In the case of legumes N_2-fixation is carried out by prokaryotes, *Rhizobium* or *Bradyrhizobium* in nodules located on the plant root (Sprent et al., 1990).

Nodulation and the development of an effective symbiosis is a complex process requiring both bacterial and plant genes. The molecular mechanisms of recognition between *(Brady)rhizobium* and legumes can be considered as a form of cell-to-cell interorganismal communication. A precise exchange of molecular signals between the host plant and rhizobia over space and time is essential to the development of effective root nodules. The first apparent exchange of signals involves the secretion of phenolic compounds, flavonoids and isoflavonoids, by host plants (Peters and Verma,

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1990). These signal compounds are often excreted by the portion of the root with emerging root hairs, a region that is most susceptible to infection by rhizobia (Verma, 1992). These compounds have been shown to activate the expression of *nod* genes in rhizobia, stimulating production of the bacterial nod factor (Kondorosi, 1992). This nod factor has been identified as a lipo-oligosaccharide (Carlson et al., 1993), able to induce many of the early events in nodule development, including deformation and curling of plant root hairs, the initiation of cortical cell division, and induction of root nodule meristems. In soybean for example, the isoflavones, daidzein and genistein, are the major components of soybean root exudates which induce the *nod* genes of *B. japonicum* (Kosslak et al., 1987). Other such substances active at very low concentrations (10^{-6} to 10^{-7} M) have been shown to stimulate bacterial *nod* gene expression within minutes. However, the effectiveness of isoflavonoids is found to vary between cultivars.

Another similarity in the nod region(s) of *Rhizobium* strains is the presence of conserved sequence elements within the promoter regions of certain inducible nod genes. These conserved sequences, first identified in the *nodABC* promoter region, are termed the nod-box and are believed to function in induced nod gene expression, possibly as regulatory protein binding sites.

No Sym plasmids have been associated with *Bradyrhizobium* strains. The nitrogenase and nodulation genes of these bacteria are encoded on the chromosome. Of importance, *Bradyrhizobium* strains contain nodulation genes which are reported to functionally complement mutations in *Rhizobium* and which show significant structural homology to nodulation gene regions of *R. meliloti* and *R. leguminosarum*.

The specific components of legume exudate that act to induce nodulation genes in several species of *Rhizobium* and *Bradyrhizobium* have been identified as flavonoids and related compounds. Luteolin was

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reported to be the component of alfalfa exudates that induces *nodABC* expression in *R. meliloti*. Three clover exudate constituents: 4',7-dihydroxyflavone, geraldone and 4'-hydroxy-7-methoxyflavone were reported to induce the nodulation genes of *R. trifolii*. Two pea exudate components: eriodictyol, and apigenin-7-O-glucoside were reported to induce the nodulation genes of *R. leguminosarum*. In addition, molecules having structures related to those of the inducer found in exudate were assessed for their ability to induce. Inducers of *Rhizobium* nodulation genes appear in general to be limited to certain substituted flavonoids, and the range of compounds to which a *Rhizobium* responds is species specific. Since host range is used to classify *Rhizobium* strains into different species, this suggests that differential response to inducer molecules is involved in the mechanism of determination of host range.

Two isoflavone components of soybean exudate, daidzein and genistein, have been reported to be inducers of the nodulation genes of *B. japonicum* strains 110 and 123 (Kosslak et al., 1987, Proc. Nathl. Acad. Sci. USA 84:7482-7432). Several other isoflavones were found to be inducers (7-hydroxyisoflavone, 5,7-dihydroxyisoflavone and biochanin A) or weak inducers (formononetin and prunetin) of the *B. japonicum nod* genes. In addition, two flavones: 4',7-dihydroxyflavone and apigenin which induce certain *Rhizobium* nod genes were also found to induce the *B. japonicum nod* genes.

In view of the above, it is clear that the exchange of signals between legume and bacterial strain and intricacies thereof are shared between different legumes and the *Rhizobium* and *Bradyrhizobium* genera. The manner in which nodulation genes are regulated is also conserved among *Rhizobium* and *Bradyrhizobium* strains.

Soybean [*Glycine max* (L.) Merr.] is the world's most widely-produced nitrogen (N) fixing crop. However, soybean is a plant of

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tropical to subtropical origin and, as such, requires temperatures in the 25 to 30°C range for optimal growth and symbiotic N_2 fixation. When well-nodulated, soybean is capable of fixing its own N. Both symbiotic N_2 fixation and NO_3^- utilization appear to be essential for maximum yield. High soybean yields also require adequate levels of phosphorous and potassium. Liming acid soils to a pH of 6.0 to 6.5 is an important prerequisite for profitable soybean production. Adequate populations of *Bradyrhizobium japonicum* must be present to produce a well-nodulated soybean crop. Smith et al. (1981) determined that an inoculum level above 1 x 10^5 rhizobia per centimetre of row was necessary to establish effective nodulation.

Root zone temperatures (RZTs) below 25°C strongly and negatively affect soybean nodulation and N_2 fixation (Lynch et al., 1994). In fact, in short season areas low temperature is considered the major growth limiting factor for soybean. It has been noted that all stages of nodule formation and functioning are affected by suboptimal RZTs and experiments have generally indicated that early nodule development processes are the most sensitive. The exact mechanism by which suboptimal RZTs affect N fixation has yet to be identified. Numerous hypothesis have been postulated however: 1) decrease N fixation activity by the nitrogenase enzyme complex; 2) changes in nodule oxygen permeability; 3) rate of export of fixed N from the nodule; 4) inhibition of N_2 fixation inside the nodule; 5) decrease in bacteroid tissue and/or delay in its rate of formation; 6) via effects on bacterial physiology and growth; and 7) via effects on plant physiology and growth.

Production of N fertilizer, in Canada as elsewhere, is economically ($1 billion per year in Canada), energetically (equivalent to 30 million barrels of oil per year) and environmentally (produce 15 million tones of CO_2 per year, ground water-polluting NO_3 and ozone-destroying NO_x) expensive. In eastern Canada the farm community spends approximately

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$150 x 10^6 per year for N fertilizer. Nitrogen fixation is the sustainable alternative to N fertilizer. Therefore, an understanding of the mechanism of suboptimal RZT effects on soybean nodulation and N_2 fixation and finding methods to reduce this restriction by low RZT would allow increased use of this N_2-fixing cash crop, and decreased reliance on potentially polluting N fertilizers in cool season areas. The ability to overcome the negative effects of suboptimal RZTs could be applied to other stress conditions that negatively affect nitrogen fixation (water stress, high pH, temperatures etc.). As well, such an overcoming of stress conditions that negatively affect nitrogen fixation, growth, yield and the like could be adapted to other legumes and possibly to non-legumes.

Understanding of rhizosphere biology has progressed with the discovery of a specific group of microorganisms, now called plant growth promoting rhizobacteria (PGPR), that can colonize plant roots and stimulate plant growth and development (Kloepper et al., 1980a). Most of the identified strains of rhizobateria occur within gram-negative genera, of which fluorescent pseudomonads are most characterized, although some strains of *Serratia* have been reported (Kloepper et al., 1986, 1991; Ordentlich et al., 1987). Several gram positive strains of root-colonizing bacteria were reported such as an *Arthrobacter*-like genus (Kloepper et al., 1990) and *Bacillus* (Backman et al., 1989; Turner et al., 1991). Other documented PGPR, include *Azotobacter* species, *Azospirillum* species, and *Acetobacter* species (Brown, 1974, Elmerich, 1984; Bashan et al., 1990; Tang, 1994).

Several reports related the beneficial effects of PGPR to direct plant growth promotion, disease suppression, associative N_2 fixation and improved access to soil nutrients. Nitrogen fixation promoting rhizobacteria are identified as PGPR that are capable of increasing nodulation as well as plant growth. A different approach was taken recently (Kloepper et al., 1986; Kloepper et al., 1988b) when over 10, 000 bacterial

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strains were randomly isolated from plant rhizospheres at different locations. After examining their ability to growth at low temperatures, they were tested under greenhouse conditions, using field soils, for their ability to promote the growth and development of soybean and canola plants. Selected strains were then tested in field trials, and some were found to increase growth and yield. However, some promising strains under lab conditions were not shown to exhibit their effect in the field. Furthermore, the effect of PGPR strains was not tested on legumes to assess whether they could somehow increase nodulation and nitrogen fixation under environmental stress conditions, such as low root zone temperatures (RZT) which retard the onset thereof. It follows that whether such an effect of PGPRs on nodulation and nitrogen fixation translated into an increase protein yield and dry matter yield of legumes grown under such environmental stress conditions was not determined.

Inoculation of crop seeds with PGPR was of major interest in agricultural production throughout the twentieth century. The commonly applied inoculants have involved nitrogen-fixing strains of *Rhizobium*, which can form a symbiotic association with legumes and fix nitrogen. The capability of soil bacteria that are free-living in the soil (non-symbiotic or "associative") to colonize roots and promote plant growth has been well documented over the past 30 years (Kloepper et al., 1988a). Initial efforts to enhance plant growth with free-living bacteria were centered on either phosphate-solubilizing bacteria such as *Bacillus megaterium* var. *phosphoticum* or on nitrogen-fixing bacteria that are present in the soil as free living bacteria, such as *Azotobacter* (Cooper, 1959; Brown, 1974). The free-living phosphate solubilizing and nitrogen-fixing bacteria were shown to increase the yield up to 25% (Schmidt, 1979). The problem with these bacteria is their inability to compete effectively with other soil microflora, as their population density decline after they have been introduced into the

rhizosphere. This observation established the "biological balance" (Baker et al., 1974) or "microbial equilibrium" (Katznelson, 1985) theory, which stated that the rhizosphere microflora are a distinct collection of organisms that can live and persist in the soil in equilibrium (Kloepper et al., 1988a). The assumption was that after new bacteria are introduced into the rhizosphere their population density will decrease, and the original microbial balance would be reestablished (Kloepper et al., 1988a).

In the late 1970s, researchers at the University of California in Berkeley found that the rhizosphere microbiological equilibrium could be modified by introducing specific strains of rhizosphere pseudomonads (Kloepper et al., 1988a). These pseudomonads were able to colonize roots and alter the balance of both fungal and bacterial microflora throughout the growing season (Kloepper et al., 1980c).

PGPR were reported to increase plant yields 10 to 30% in non-legume crops such as potato, radish, and sugar beet. Numerous reports indicated that PGPR can exert precise effects on diverse hosts, including lentil (Chanway et al., 1989), peanut (Turner et al., 1991), bean (Anderson et al., 1985), canola (Kloepper et al., 1988b), cotton (Backman et al., 1989; Greenough et al., 1989,), pea (Chanway et al., 1989), rice (Sakthivel et al., 1987), and soybean (Polonenko et al., 1987). The mechanism by which the PGPR promote plant growth is unknown; however, a wide range of mechanisms were postulated such as: mobilization of insoluble nutrients (e.g. phosphate) and resulting enhancement of uptake by the plant (Lifshitz et al., 1987), associative nitrogen fixation (Chanway et al., 1991a) production of antibiotics toxic to soil-borne pathogens (De-Ming et al., 1988), production of plant growth regulators that promote plant growth (Kloepper et al., 1981b; Gaskins et al., 1985; Neilands et al., 1986), siderophore production [high-affinity iron (III) chelator]. Specific Pseudomonad strains have established yield increases, control of soil-borne plant pathogens, promotion

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of seedling emergence, and promotion of legume nodulation by nitrogen-fixing *(Brady)rhizobium* spp under field conditions.

Some reports suggested beneficial effects of some PGPR on the legume N_2-fixing symbiosis; the bacteria involved are known as nodule promoting rhizobacteria (NPR). Inoculation with NPR, often pseudomonads, and *(Brady)rhizobium* enhances root nodule number or mass (Singh et al., 1979; Burns et al., 1981; Grimes et al., 1987; Polonenko et al., 1987; Yahalom et al., 1987). The ability of NPR to increase nitrogenase activity was also documented (Iruthayathas et al., 1983; Alagawadi et al., 1988). Burns et al. (1981) reported that co-inoculation of *Azotobacter vinlandii* and *Rhizobium* spp. increased the numbers of nodules on the roots of soybean (*Glycine max*), pea (*Vigna unguiculata*), and clover (*Trifolium repens*). Both field and greenhouse data showed that co-inoculation of *Pseudomonas putida* increased nodulation of beans (*Phaseolus vulgaris*) by *R. phaseoli* (Grimes et al., 1984). The mechanism by which NPR increase nodulation and/or N_2 fixation is obscure and the ecology of NPR is poorly understood.

Different mechanism have been proposed to explain how rhizobacteria promote plant growth:

(1) by producing plant growth regulators (PGRs), which are organic substances that, at very low concentrations, can influence physiological mechanisms of the plants. Several soil microorganisms have the capability to produce active quantities of PGRs, which can effect plant growth and development. Production of PGRs has been illustrated in both culture media and in the soil. Plant growth promoting activity by soil microflora can take different forms such as, the production of phytohormones, or fungicidal or bactericidal indole and phenols. *Azospirillum*, which is a diazotrophic bacterium associated with plant roots, particularly with forage grasses and cereals, in tropical regions, might be of agronomic importance, since it has repeatedly been reported to promote plant growth (Okon, 1994). It is thought

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to benefit plants by the production by phytohormones, e.g. auxins (Hartmann et al., 1994).

(2) by enhancing phosphate uptake by plants. Considerable research efforts have been aimed at evaluating phosphate-solubilizing bacteria. Several ways by which these bacteria, which include fluorescent pseudomonads, may increase the availability of phosphorus to plants were suggested such as, mineralization (solubilization of organic phosphate via the action of phosphatase) or solubilization of unavailable inorganic phosphates by means of organic acids. Lifshitz et al. (1987) reported that a *P. putida* PGPR strain increased the uptake of ^{32}P-labelled phosphate by canola seedlings. Inoculation of seeds with a pseudomonad PGPR resulted in a significant increase of ^{32}P levels in roots and in shoots.

(3) By enabling a biological control of soil-borne plant pathogens. Phytophathogens can reduce crop yields by 25-57%, which is tremendous loss of crop productivity. Presently, chemical agents (pesticides) are applied to reduce this loss. Other procedures such as fumigation, steam treatment, and solarization of soils were also applied (Gamliel et al., 1992). However, many of these chemicals could have hazardous effects on animals, including humans, and may persist and accumulate in natural ecosystems. Currently, biological approaches are being developed to control some plant pathogens. Such biological approaches include the development of plants that are able to resist one or more pathogenic agents (Greenberg et al., 1993) and the use of PGPR that can suppress or prevent the phytopathogenic damage (O'Sullivan et al., 1992; Sivan et al., 1992; Cook, 1993; Sutton et al., 1993).

The possible use of PGPR as biological control agents is described by the study of the mode of action for fluorescent pseudomonad PGPR reported for potato (Kloepper et al., 1981a). These PGPR strains were not able to promote plant growth under gnotobiotic conditions (Kloepper et

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al., 1981b). The growth promotion in field soils was accompanied with a 23-64% reduction in the population densities of indigenous rhizoplane fungi and a 25-93% reduction in gram-positive bacterial population densities (Kloepper et al., 1981a). Suslow et al. (1982) identified specific strains of root-colonizing bacteria that were pathogenic on sugar beet seedlings and were termed deleterious rhizobacteria (DRB). Several genera of DRB were found to cause growth inhibition and root deformations on crop plants (Suslow et al., 1982; Fredrickson et al., 1985a; Fredrickson et al., 1985b; Gerhardson et al., 1985; Campbell et al., 1986; Schippers et al., 1987;).

Most PGPR strains appear to enhance plant growth indirectly by reductions in populations of DRB. A study by Kloepper (1983) demonstrated that inoculation of potato seed pieces with two strains of fluorescent pseudomonad PGPR, which were responsible for yield increases in the field, caused a reduction in populations of *Erwinia carotovora* on roots, ranging from 95 to 100% fewer than controls without PGPR treatment. Colyer et al. (1986) and Xu et al. (1986a,b) confirmed the biological control of *E. carotovora* by selected strains of root-colonizing fluorescent pseudomonads.

A number of such mechanisms by which rhizobacteria demonstrate biological control have been identified. Generally, bacterial abilities to protect the plants from soil-borne plant pathogens rely on two aspects: the root-colonization capacity of the biocontrol agent and the production of siderophores and antibiotics that control the growth of the plant pathogens. Such mechanisms include:

(1) the production of siderophores. PGPR produce and release siderophore molecules that will bind most of the Fe^{+3} that is available in the rhizosphere, and as a result, prevent any pathogens from proliferating because of lack of iron, and thus facilitate plant growth (O'Sullivan et al., 1992). Pseudomonad PGPR strains are capable of producing siderophores which chelate the ferric iron in the rhizosphere thus inhibiting plant pathogenic or deleterious species,

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with less affinity for iron (Kloepper et al., 1980b). An awareness of the behaviour of introduced PGPR strains and their siderophore-producing abilities at different temperatures could be of major importance regarding the use of PGPR for promotion of plant growth. However, the influence of PGPR strains on plant growth under different climatological conditions needs additional study before PGPR strains can be introduced into the field.

(2) The production of antibiotics.

(3) Competition among soil microorganisms for infection sites and nutrients.

(4) Hydrolysis of fusaric acid (Toyoda et al., 1991).

(5) Synthesis of enzymes that are able to hydrolyze the cell walls of fungal pathogens (Mauch et al., 1988).

It has also been reported that enhancement of plant growth by rhizobacteria through disease control may comprise direct or indirect effects on the pathogen (Davison, 1989). Directly by competing with the pathogen for available nutrients, siderophore production, and production of antibiotics (Weller, 1988), and indirectly by modifying plant defence responses. Induced disease resistance is an active resistance mechanism which depended on the host plant's physical or chemical restrictions, activated by biotic or abiotic agents (Kloepper et al., 1992).

It is generally understood that root colonization is a dynamic process and not a temporary relation between bacteria and roots in soil. It is the process whereby bacteria survive inoculation onto seeds or into soil, divide and proliferate in response to seed exudates rich in carbohydrates and amino acids (Kloepper et al., 1985), adhere to the root surface (Suslow, 1982; Weller, 1983), and colonize the root system in soils containing native microorflora (Kloepper et al., 1980a, Suslow and Schroth, 1982; Weller, 1984). PGPR can multiply and remain in the rhizosphere following inoculation onto crop seeds. The bacteria are allocated in the rhizosphere in a log normal order (Loper et al., 1984) and are sporadically established along the roots

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(Bahme et al., 1987). Colonization, although difficult to measure, is required for causing an interaction with the plant and other members of the microflora. The role of root colonization by PGPR has been reviewed (Schroth et al., 1982; Suslow, 1982).

Rhizosphere colonization symbolizes a larger ecological niche including root colonization and bacteria that are in close proximity to, although not necessarily attached to roots (Kloepper et al., 1980a). Fluorescent pseudomonads, are highly rhizosphere competent (capable of root colonization), which accounts for their predominance among the PGPR. Pseudomonads hold several characteristics which assist them in seed colonization, such as fast growth and motility (Seymour et al., 1973; Arora et al., 1983; Scher et al., 1985;). However, these traits may not always correlate with root colonization. For example, Howie et al. (1987) found that three nonmotile mutants of *P. fluorescens* colonized wheat roots as well as their motile parents.

Rhizosphere colonization has been reviewed recently by van Elsas et al. (1990), Kloepper et al. (1992) and Kluepfel (1993). The major problem for successful application of PGPR strains in soil was suggested to be the lack of constant effectiveness of the inoculant (van Elsas et al., 1990). Several causes were suggested, such as ineffective colonization of the plant, or poor survival of the introduced population. Xu et al. (1986b) and Bull et al. (1991), demonstrated a positive relationship between root colonization by a PGPR strain and disease suppression, proposing that methodologies which enhance root colonization may also improve the benefits of a PGPR strains in soil. The extent and amount of root colonization required by a PGPR strain in order to enhance plant growth rely on many interrelated considerations. The choice of methods used to try to increase rhizosphere colonization and plant growth should take these factors into account (Stephens, 1994b).

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As the beneficial bacteria are introduced into the rhizosphere, they become involved in a web of complex biological interactions with the host plant and with the surrounding rhizosphere microorganisms. They obtain their nutrients from the root exudates and are accordingly dependent on the host plant, while they affect the host by inducing physiological changes in the plant (Kloepper et al., 1988b). Interactions with indigenous rhizosphere microorganisms could take different forms. It could be neutral, antagonistic (e.g. competition for nutrients, production of antibiotic compounds, parasitism, or predation) or synergistic (i.e. the promotion of *Rhizobium*-induced nodulation of legumes). Several environmental restrictions, including temperature, moisture, and soil type, may affect these microbial interactions.

In view of the intrinsic complexity of the PGPR strains themselves, of the complex relationship with its environment, of the complexity and unpredictability of environmental factors affecting the environment, the selection of a particular strain to be tested in the field is at best an educated guess. Whether a chosen PGPR will survive, colonize the root and affect growth, nodulation, nitrogen fixation and yield cannot *a priori* be predicted.

Nevertheless several such factors can be used in order to increase the chance of selecting rhizosphere competent strains. Such factors include: (1) Crop specificity; (2) location on the root; (3) quantity of inoculum on the seed; (4) co-inoculation of PGPR strains with other microorganisms; (5) use of carrier materials to improve PGPR survival in the rhizosphere; and (6) management of the soil.

The ecology of pseudomonad PGPR is a relatively new research area. Consequently, there is little understanding of how environmental factors will influence bacterial colonization effects and persistence on roots and the resulting effects on plant growth. Thus, the

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potential effects of specific PGPR strains on certain legumes, as assessed by increased nodulation, nitrogen fixation, and yield, based on laboratory testing can clearly not be directly transposable to the complex field situation.

PGPR ability to increase crop yields under diverse field conditions has nevertheless been reported (Schroth et al., 1982; Suslow, 1982; Hemming, 1986; Schippers et al., 1987; de-Freitas et al., 1992). However, some of these reports demonstrated that seed inoculation with PGPR does not always lead to yield increases. Possible reasons for this disagreement, including iron availability, soil-type, and the nature of the soil microflora and application procedures have already been discussed.

Other field work has explored the enhancement of seedling emergence. That specific root-colonizing bacteria can increase seedling emergence was first reported with strains that caused increases in emergence rates of soybean and canola seedlings under cold field conditions in Canada (Kloepper et al., 1986). The new class of PGPR strains was termed emergence-promoting rhizobacteria (EPR). Inoculation of conifer seeds with *Bacillus* strains caused increased seedling emergence and biomass (Chanway et al., 1991b). Chanway (1995) also reported that seed inoculation with *Bacilus polymyxa* can result in colonization of western hemlock root systems and increase seedling emergence.

With respect to the promotion of nodulation of legumes, it was shown that specific pseudomonad strains were able to stimulate nodulation of leguminous crops by *Rhizobium* and *Bradyrhizobium*. Grimes et al. (1987) reported that a *P. putida* strain (M17) increased *Rhizobium* nodulation of bean in field soils. Similarly, Polonenko et al. (1987) tested the effects of fluorescent pseudomonads on nodulation of soybean roots by *B. japonicum*. However, the effect of low RZT or similar environmental stress factors were not assessed. In Canadian Patent 1,328,238, Polonenko et al. (1994) disclose certain PGPR strains which are apparently capable of

enhancing *B. japonicum* nodulation and soybean plant growth in field soil in pots, and thus performed under laboratory conditions. These strains were termed nodulation-promoting rhizobacteria (NPR). Although one field experiment is reported, there is no teaching or suggestion of the effect of NPR strains in environmental stress conditions such as low RZT; there also is no tracking or suggestions of the effect of PGPR on nodulation or nitrogen fixation of legumes under field conditions.

Canadian Patent 1,335,363 (1995) to Kloepper et al. discloses emergence-promoting rhizobacteria. Such strains, mostly *Pseudomonas* strains but also *Serratia* strains (including 1-102) are said to induce an increase in seedling emergence when soil temperatures were below 20 degrees centigrade. The results presented in 1,335,363 do not show increase nodulation and nitrogen fixation, and moreover, they are not based on field data. Hence, the reported effect of specific PGPR strains on seedling emergence of legumes cannot be transposed to the complex field situation.

Canadian Patent 1,328,238 (1994) to Kloepper et al. disclose PGPR strains (mostly *Pseudomonas* strains but also *Serratia* strains including *S. proteamaculans* strain 1-102 and *S. liquefaciens* strain 2-68) that promote an increase in yield and nodulation under laboratory conditions. 1,328,238 does not teach that the disclosed PGPR act to increase nodulation and nitrogen fixation under field conditions and/or under stress conditions such as low RZT.

Due to the number of benefits which can result from the establishment of rhizobia:legume symbiosis, other strategies have been devised to promote nodulation of legumes.

US patent 4,878,936 to Handelsman et al., teaches a method for enhancing nodulation of legumes which includes inoculation in the immediate vicinity of the roots thereof, an effective quantity of bacteria which

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enhance nodulation. However, the results are based on controlled laboratory conditions, not on field studies. Moreover, the laboratory conditions used, involved temperatures above 25°C which are not expected to be limiting for nodulation.

US patent 5,141,745 to Rolfe et al., discloses flavones, some of which are leguminous plant exudates, which induce expression of certain *nod* genes in rhizobium strains. Rolfe et al., however, do not assess whether their results, all obtained under laboratory conditions, translate into increase nodulation and growth of the leguminous plant under field conditions.

The art is replete with examples demonstrating that results obtained under the laboratory setting are not predictive of the field situation. Typically, a good controlled environment provides optimal levels of soil nutrients, soil pH, soil moisture, air humidity, temperature and light. The plants are usually widely spaced so that they do not compete for light. In some cases environmental factors such as carbon dioxide may even be optimized. The field environment is vastly more complicated than that of the controlled environment setting. The soil will vary in its chemistry and texture in a fractal pattern, such that, while the soil of a research site can be characterized in general, it will be variable at every level within the confines of the experimental area. In a controlled environment setting plants are usually produced in sterilized rooting media (pasteurized soil, sterile sand, or some form of artificial rooting media) and there is no soil micro flora or fauna. Field soil is an ecosystem; it contains an enormous number of bacteria, fungi, protista, algae, and soil insects. The climate and related atmospheric factors (light intensity, relative humidity, temperature, rainfall, carbon dioxide concentration of the air, presence of pollutants) vary constantly under unpredictable field conditions. Thus, for instance, a researcher may impose a nutrient limitation in the field, but if the conditions are dry and water is more

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limiting to plant growth than the nutrient in question, there will be no discernable effect due to nutrient treatments.

The inability to extrapolate from a laboratory to a field setting is illustrated by work conducted in the 1970's and early 1980's on soybean with strains of *Bradyrhizobium japonicum* which were hypothesized to be more energy efficient when fixing nitrogen. Because of the extreme stability of the triple bond in the dinitrogen molecule nitrogen fixation was known to be a very energy expensive process (reviewed in Schubert 1982). In addition, it was discovered that the enzyme which fixed dinitrogen into biologically useful ammonia (nitrogenase) leaked high energy electrons to protons, so that every time one dinitrogen molecule was fixed into two ammonia molecules, at least one dihydrogen (the product of two protons plus two electrons) was produced. This constituted a waste of energy by the plant-bacterium symbiotic system. Shortly afterward it was discovered that some strains of *B. japonicum* contained an enzyme that took up the hydrogen formed and took the high energy electrons back off the protons, hence recovering much of the energy that would have been lost (Schubert et al. 1978). This lead to speculation that strains containing these "uptake hydrogenases", referred to as Hup+ strains, would be more efficient and lead to improved plant growth, as the plant would have to supply less energy (as organic acids) to the bacteria for each ammonia molecule received from them. Albrecht et al. (1979) compared soybean plants inoculated with Hup+ and Hup- strains of *B. japonicum* under greenhouse conditions. Average total nitrogen contents and total dry weights of Hup+ inoculated plants were shown to be larger than those of plants inoculated with Hup- strains. This was confirmed by Maier et al. (1978). However, under field conditions, Albrecht et al. (1979) were unable to detect an increase in dry matter production or yield between Hup+ and Hup- strains. These results were confirmed by numerous field condition studies. During the course of these confirmations

however, a superior strain of *B. japonicum* (532c), which is now included in almost all soybean inoculants used to produce soybean in Canada, was identified (Hume et al., 1990). Strikingly, this strain is Hup-.

This example provides a blatant proof involving soybean, that results obtained in a controlled milieu are *a priori* not predictive of the field situation.

PCT patent application WO 94/25568, which was published November 10, 1994 in the name of Rice et al., discloses cold tolerant strains of *Rhizobium* which are useful for improving nodulation, nitrogen fixation and overall crop size under field conditions. However, it is unclear whether the cold-selected strains indeed provided an advantage to alfalfa, since in certain experiments the temperate strains performed better than the cold-temperature selected strain (i.e. Tables 5,6 and 7). This results corroborates the findings of Lynch et al., 1994 which suggested that inoculation with *B. Japonicum* strains from cold environments is unlikely to enhance soybean N_2-fixation under cool soil conditions. Lynch et al., 1994 also suggested that indeed the host plant, and not the bacterial strain, mediates at least a significant portion of the sensitivity of N_2-fixation under low RZT. Further WO 94/25568 (see below) teaches that commercial rhizobial inoculants are not consistent in their efficacity and performance, and nodulation failures after use of commercial inoculants are common. This is explained by the inability of inoculant strains to out-compete indegenous rhizobial bacteria for root-infection sites, once again demonstrating the non-predictivity of laboratory results to the field conditions.

US Patent 5,432,079 to Johansen et al., relates to the isolation of *Rhizobium* strains having improved symbiotic properties. Once again this Patent fails to teach an enhancement of growth and or yield of a legume under field conditions. Moreover, this document is silent on the use of flavonoids or the like to achieve that goal. It is also silent on the use of

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PGPRs. It teaches however that a higher expression of the *nod* genes does not necessarily provide an advantage, but can be detrimental to the competitive ability of the *Rhizobium* strains.

It should be noted that to yield maximally a legume which is dependant on nitrogen fixation requires both the correct amount of nodulation and the correct type of nodules. In general, the best situation for the plant is to generate a small amount of nodule tissue which is extremely efficient at nitrogen fixation. If, for some reason, a given crop legume, is unable to form sufficient nodule tissue, of forms inefficient nodule tissue, its yields will be lower than would other wise be the case. During the 1960's and early 1970's there was extensive research on the genetics of nitrogen fixation, from both the bacterial and the plant perspectives. Numerous strains of (Brady)Rhizobium which formed nodules but fixed little or no nitrogen were characterized (reviewed extensively in Grant et al., 1973). This could result in nodule tissue being a larger proportion of the total legume biomass, but lower legume yield. At a less dramatic level, a study by Lynch et al. (1993) showed that some strains of Bradyrhizobium produce larger amount of soybean nodule mass than others, and that some of the "better nodulators" increased soybean growth, but, some did not. Some strains appeared to produce more nodule tissue with the same level of nitrogen fixation, while other strains increased the amount of nodule tissue formed, but this extra nodule tissue was less efficient than was the case with some other strains which produced less nodule mass.

Finally, legume plants can produce too much nodule tissue, even if it is efficient. Carroll et al. (1985) characterized supernodulating mutants. These mutants form substantially more nodule tissue and fix substantially more nitrogen than normal legumes, however, this is a situtation which is out of balance. So much (too much) of the plants energies are directed toward nitrogen fixation that the growth and yield of these plants is

less than those normal for the crop types (Carroll et al., 1985; Vest et al., 1973).

To date there has been no investigation as to whether nodulation inhibiting or delaying factors, such as suboptimal RZTs alter the effect of PGPR on legumes under field condition. Further, there has been no investigation as to a possible compensating role of PGPR on nodulation, nitrogen fixation and yield under short season conditions.

Elucidation of the mechanisms which affect nodulation and nodule formation in soybean (or other legumes) under environmental stress factors, such as suboptimal RZTs, and a determination of how to reduce the negative effects of such stress factors on N_2 fixation and symbiosis under conditions which inhibit or delay this symbiosis would provide a significant advantage to the production of legumes. For example, it would be advantageous to understand whether the poor nodulation of soybean at suboptimal RZTs can be compensated for by the use of PGPR.

There thus remains a need to reduce the negative effects of environmental factors on nodulation and nodule formation and to provide compositions and methods to enable the enhancement of grain yield and protein yield of legumes grown under environmental conditions that inhibit or delay nodulation thereof.

The description found herein refers to a number of documents, the content of which is herein incorporated by reference.

Recent reviews on nodulation factors and *Rhizobium* symbiosis are available: Spaink (1995) and Prome et al. (1996).

SUMMARY OF THE INVENTION

The Applicant was the first to demonstrated that PGPR can compensate for the retardation of nodulation and nitrogen fixation at low RZTs or under short season conditions. The applicant is also the first to

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demonstrate that PGPR addition can affect early stages in the complex process leading to nitrogen fixation, under low RZTs.

Before the present invention there had been no investigations as to whether an inhibitor of the early steps of nodulation, such as suboptimal RZTs could be overcome by the addition of PGPR under field conditions. The Applicant then showed that this technique, applied into the plant rhizosphere, accelerates the onset of soybean nitrogen fixation and increases total seasonal fixed nitrogen under field conditions in a short season area. The results of these experiments further indicated that PGPR addition increases protein and dry matter yield under short season conditions. Surprisingly, nine PGPR strains which had been selected in cold environments were tested in the laboratory to verify whether they could compensate the inhibitory effect of low RZTs, only three strains did. These three were thus tested in the field and only two demonstrated a potential in increasing nodulation, nitrogen fixation and yield under field conditions.

Thus in a first aspect, the present invention features compositions comprising PGPR for enhancing nodulation of legumes grown under environmental conditions which inhibit or delay nodulation thereof. Also the present invention features compositions comprising PGPR for enhancing protein yield and grain yield of legumes grown under environmental condition which inhibit or delay nodulation thereof. More particularly, the present invention features compositions comprising a PGPR strain of the genus *Serratia* and even more particularly of compositions comprising a PGPR strain of *Serratia liquefaciens* and *Serratia proteamaculans*. In a particular embodiment, the invention features PGPR strains *Serratia liquefaciens* 2-68 and *Serratia proteamaculans* 1-102.

In another aspect, the present invention features compositions for enhancing nodulation and/or enhancing yield of legumes grown under environmental conditions which inhibit or delay nodulation

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thereof comprising at least one strain of PGPR, and more particularly at least one strain of *Serratia*.

In a related aspect, the invention features methods for enhancing nodulation of legumes grown under environmental condition which inhibit or delay nodulation thereof, the method comprising an inoculation of PGPR. Also, the present invention features methods for enhancing protein yield and grain yield of legumes grown under environmental condition which inhibit or delay nodulation thereof, the method comprising an inoculation of PGPR.

In one preferred embodiment, the present invention features compositions and methods for enhancing protein yield and grain yield of soybean grown under environmental condition which inhibit or delay nodulation thereof. In certain embodiments, co-inoculation of soybean with PGPR and rhizobacteria is disclosed.

In accordance with the present invention, there is provided a composition for enhancing nodulation of a legume grown under environmental conditions that inhibit or delay nodulation thereof, the composition comprising an agriculturally effective amount of a PGPR with a suitable carrier medium.

In accordance with the present invention, there is also provided a method for enhancing nodulation of a legume grown under environmental conditions that inhibit or delay nodulation thereof, comprising:

a) inoculating in the vicinity of one of a seed and root of said legume with a composition comprising an agriculturally effective amount of a PGPR with a suitable carrier medium.

In accordance with the present invention, there is also provided a composition for enhancing grain yield and protein yield of a legume grown under environmental conditions that inhibit or delay nodulation

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thereof, the composition comprising an agriculturally effective amount of a PGPR with a suitable carrier medium.

In accordance with the present invention, there is also provided a method for enhancing grain yield and protein yield of a legume grown under environmental conditions that inhibit or delay nodulation thereof, comprising:

a) inoculating in the vicinity of one of a seed and root of said legume with a composition comprising an agriculturally effective amount of a PGPR with a suitable carrier medium.

In accordance with the present invention, there is further provided a composition for enhancing the rate of emergence of a legume comprising an agriculturally effective amount of a PGPR strain with a suitable carrier medium. In a preferred embodiment, the composition for enhancing the rate of emergence of a legume further comprises a cytokinin such as kinetin.

While the instant invention is demonstrated mostly by experiments performed with *Bradyrhizobium japonicum* and soybean, the invention is not so limited. Other legume crops, and rhizobial strains may be used using the same principle taught herein. Non-limiting examples include, alfalfa, *Rhizobium meliloti* and a *nod* gene inducing factor thereof; clover *R. meliloti*, and a *nod* gene inducing factor thereof; clover *R. trifolii*, and a *nod* gene inducing factor thereof; *R. leguminosarum* peas or lentils, and a *nod* gene inducing factor thereof; and beans *R. phaesoli* and a *nod* gene inducing factor thereof. Preferred matching of *Rhizobium* species with legume crop groups include:

Rhizobial species	Legume crop group
Rhizobium melilotti	alfalfa, sweet clover
Rhizobium leguminosarum	peas, lentils
Rhizobium phaesolii	beans

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Bradyrhizobium japonicum	soybeans
Rhizobium trifolii	red clover

It will be understood that the composition of the present invention could contain other stimulating factors such as a number of flavonoids, isoflavonoids, flavones including flavanones, flavanols and dihydroflavanols, isoflavones, coumarins kinetin, gibberellic acid and related molecules.

Nodulation inducing activity was found to reside in a structurally identifiable group of compounds not limited to those flavones associated in particular with legumes which include specifically substituted flavones, flavonones (dihydroflavones), flavanols (3-hydroxyflavones) and dihydroflavanols. Thus, numerous flavones and dihydroflavanols have nodulation inducing activity (Hungria et al., 1997; Stacey et al., 1995; Spaink, 1995). Although alkoxy substituted flavone other than methoxy have not been identified from natural sources, there is no reason to believe that alternative short chain substituents like ethoxy or propoxy groups would abolish nodulation gene induction activity.

Synthetic as well as natural nodulation gene-inducing compounds are encompassed by the scope of the present invention.

Direct or indirect methods of legume inoculation can be employed. During direct inoculation the bacterium is applied directly to the seed prior to sowing. This can most simply be accomplished by spraying the seed with or dipping the seed into a liquid culture containing a desired PGPR strain.

The concentration of the inoculum will be adapted to the particular situation at hand by the skilled artisan. For example, the skilled artisan will take into account the level of severity of inhibition or delay of the environmental conditions on nodulation, the intrinsic activity of a chosen PGPR, the method of application of the composition, tc.

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Hereinafter, the invention is illustrated with reference to *B. japonicum* and soybean, but is demonstrative of utility of the invention with other *Rhizobium* and *Bradyrhizobium* species and other legume crop groups. Indeed, herein the term rhizobia is used loosely and encompasses *Rhizobium* and *Bradyrhizobium* species.

The term "PGPR" refers to free living rhizobacteria that, in the absence of an intimate symbiotic relationship, enhance nodulation, nitrogen fixation, growth, yield and the like of legumes grown under field conditions.

The term "environmental conditions which inhibit or delay nodulation" should be interpreted herein as designating environmental conditions which postpone or inhibit nodulation and nitrogen fixation and include, without being limited thereto: conditions that stress the plant, such as temperature stress, water stress, pH stress as well as inhibitory soil nitrogen concentrations or fixed nitrogen.

As used herein, the recitation "low root zone temperature (RZT)" as well known to the person of ordinary skill, relates to a temperature below the optimum temperature of growth, nitrogen fixation (if applicable), yield and the like of a chosen crop. This optimum temperature of course varies from crop to crop, and thus so does the low root zone temperature. In the case of soybean for example which has an optimal temperature for nodulation and growth between 25°C and 30°C, low RZT is thus any temperature below about 25°C. It will be understood to the person of ordinary skill that the above-mentioned conditions which delay nodulation and/or growth of a specific crop will also vary according to the crop and are conditions which fall outside the optimum range for growth and/or nodulation of the specific crop. These specific optimum ranges of conditions are well known in the art. Of course, it will be recognized by the person of ordinary

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skill that a further deviation from the optimum temperature (below 17°C in the case of soybean) can drastically affect growth and nodulation.

As used herein, the term "enhancing protein yield and grain yield" refers to an enhancement of protein and grain yield of legumes of treated plants in accordance with the present invention or adaptations thereof as compared to control plants.

"An agriculturally effective amount of a composition" for increasing the growth of legume crops refers to a quantity which is sufficient to result in a statistically significant enhancement of nodulation and/or nitrogen fixation and/or growth and/or of protein yield and/or of grain yield of a legume crop as compared to the enhancement of nodulation and/or nitrogen fixation and/or growth, protein yield and grain yield of a control crop.

The term "immediate vicinity of a seed or roots" refers to any location of a seed or roots wherein if any soluble material or composition is so placed, any exhibit of the plant or of the bacteria, or bacterial cells will be in actual contact with the seed as it germinates or the roots as they grown and develop.

The recitation "root associated PGPR" is defined herein as the microbial population on both the root and the soil attached to the root, while "rhizosphere" is about 5-10 cm region around the plant root where materials released from the root increase the microbial population and its activities (Prescott et al., 1993). In accordance with the present invention, from about 10^4 to about 10^{10} PGPR cells per ml should be inoculated, preferably between about 10^6 to about 10^8 and more preferably about 10^8 cells per ml.

The term "Kinetin" refers to a plant growth regulator which is a common member of the group of compounds known as cytokinins, having a core molecule which is adenine-like (Salisbury et al., 1992). Non-limiting examples of other cytokinins having similar plant growth

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regulating activities and encompassed within the scope of the present invention include zeatin, benzyladenine and isopentyladenine. The concentration of cytokinin such as kinetin to use in accordance with the present invention range between about 0.1 µM to about 20 µM, preferably between about 1 µM to about 10 µM, and more particularly between about 3 µM to about 8 µM.

By "nodulation gene-inducing" or "*nod* gene-inducing" is meant bacterial genes involved in nodule establishment and function.

Representative examples of PGPR strains were deposited at the ATCC, 10801 University Boulevard, Manassas, Va. PGPR strain of *Serratia proteamaculans* 1-102 and *Serratia Liquefaciens* 2-68 were granted ATCC accession numbers _____ and _____, respectively.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non restrictive description of preferred embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

Figure 1 shows the effects of Kinetin concentrations on total plant dry matter one month after emergence;

Figure 2 shows interaction of Kinetin and PGPR on nodule number at one month after emergence;

Figure 3 shows the effect on Kinetin concentrations on nodule weight at one month after emergence;

Figure 4 shows the effects of Kinetin concentrations on nodule number at early pod stage (R3);

Figure 5 shows the interaction of cultivars and the Kinetin concentration on nodule weight at early pod stage (R3);

Figure 6 shows the interaction of cultivars and Kinetin on root weight at early pod stage;

Figure 7 shows the effect of cultivars and Kinetin on plant height at final harvest;

Figure 8 shows the effect of Kinetin on 100-seed weight;

Figure 9 shows the effect of cultivars on seed numbers and pod numbers;

Figure 10 shows the effect of cultivars on 100 seed weight;

Figure 11 shows the effect of interaction of Kinetin and PGPR on soybean grain yield.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

In order to test whether PGPR can compensate for the retardation of nodulation and nitrogen fixation under environmental stress conditions and to test whether PGPR have an utility in promoting nodulation, nitrogen fixation, increasing yield and the like of legumes when grown under field conditions, a number of field tests were carried out.

EXAMPLE 1

Plant growth promoting rhizobacteria accelerate nodulation and increase nitrogen fixation activity by field grown soybean [*Glycine max* (L.) Merr.]

Two field experiments were conducted on two adjacent sites in 1994 to evaluate the ability of two PGPR strains to increase nodulation, nitrogen fixation, and total nitrogen yield by two soybean cultivars under field conditions in a short season area. The results of these experiments indicated that co-inoculation of soybean with *B. japonicum* and PGPR increased soybean nodulation and hastened the onset of nitrogen fixation during the early soybean growing season, when the soils were still cool. As a result of the increase in these variables, total fixed nitrogen, fixed nitrogen as a percentage of total plant nitrogen, and the nitrogen yield also increased due to PGPR application. Inoculation with PGPR only also increased soybean nodulation and nitrogen fixation by native *B. japonicum*.

The stimulation of PGPR on legume symbiotic N fixation and plant growth can be affected by environmental factors, such as RZT. Under controlled environment conditions, the effect of the PGPR on soybean nodulation and N fixation (see Example 3), and plant growth and physiological activities (Zhang et al., 1996b) varied with RZT. However, to date, there have been no investigations of whether co-inoculation of *B.* japonicum with PGPR increases soybean nodulation and N fixation under short season field conditions. Therefore, in this study, we tested the hypotheses that under short season field conditions: 1) co-inoculation of *B. japonicum* with PGPR increases soybean nodulation and N_2 fixation when soybean is planted into cool spring soils, 2) final plant N and protein yield also increase due to improvement of soybean nodulation and N_2 fixation, 3) inoculation with PGPR only, in the presence of native soil *B. japonicum,* increases soybean nodulation and nitrogen fixation.

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MATERIALS AND METHODS

Field layout

The two experiments were conducted at the Emile A. Lods Research Centre, McGill University, Macdonald Campus, Montreal, Canada. Both of the experiments were performed at each of two adjacent sites. One site was sterilized with methyl bromide (50 g m^{-2}) under a plastic canopy for 72 h (sterilized site). Three days elapsed between removal of the fumigation canopy and planting. The other site was kept unsterilized. The sterilized site was included in this study to prevent possible competition from native *B. japonicum* or interference from other elements of the soil microflora that might obscure PGPR effects. The first experiment was designed as a 3 x 2 x 2 factorial organized in a randomized complete block split-plot with four replications. The main-plot units consisted of PGPR strain applications (no-PGPR application as a control, *Serratia liquefaciens* 2-68 and *Serratia proteamaculans* 1-102). These two strains were chosen based on the reports of Zhang et al. (see Example 3 and 1996b). The two soybean cultivars (Maple Glen and AC Bravor) and the strains of *B. japonicum* [532C (Hume et al., 1990) and USDA110] formed the sub-plot units. In the second experiment, two factors were tested, PGPR application and soybean cultivars with the same design as in experiment 1, except that the two soybean cultivars were the subplot units. At the sterilized site, each sub-plot was 1.6 x 2 m and consisted of three rows of plants with 40 cm between rows. At the unsterilized site, each sub-plot (2 x 3 m) consisted of four rows of plants with 40 cm between rows. The space between plots was 80 cm and between replications 1 m.

For each replication of both experiments one plot of a non-nodulating Evans was included for calculation of soil nitrogen availability and seasonal N_2 fixation. The soil type at both sites was a Chicot light sandy loam. In the previous year, 1993, this experimental field had been planted

with oat and barley, while in 1992 it was used to produce a crop of green manure alfalfa. The average nitrogen accumulation in the non-nodulating control plants was 167 kg ha^{-1}. Potassium and phosphate were provided by the spring application of 340 kg ha^{-1} of 5-20-20 according to soil test recommendations.

Inoculum preparation

In experiment 1, the inoculum was produced by culturing *B. japonicum* strains 532C and USDA110 in yeast extract mannitol broth (Vincent, 1970) in 2000 mL flasks shaken at 125 rpm at 25°C. The PGPR strains were cultured in *Pseudomonas* media (Polonenko et al. 1987) in 2000 mL flasks shaken at 250 rpm at room temperature (21-23°C). After reaching the stable phase (7-days for *B. japonicum* and 1.5-days for PGPR), both *B. japonicum* and PGPR were subcultured. When the subculture reached the log phase (3-days for *B. japonicum* and 1-day for PGPR), each of the *B. japonicum* and PGPR strains was adjusted with distilled water to an A_{620} (*B. japonicum*) and A_{420} (PGPR) value giving a cell density of 10^8 cells mL^{-1}, respectively. Equal volumes of *B. japonicum* and PGPR cultures were mixed and allowed to stand for approximately half an hour at room temperature without shaking.

Planting methods

Seeds of the soybean cultivars 'Maple Glen' and 'AC Bravor' were surface-sterilized in sodium hypochlorite (2% solution containing 4 mL L^{-1} Tween 20), then rinsed several times with distilled water (Bhuvaneswari *et al.*, 1980). These cultivars were selected as they have been developed for production under the short season, cool conditions of eastern Canada and have performed well there. The seeds were planted by hand on May 11 and 18 at the unsterilized and sterilized sites, respectively. The delay in planting the sterilized site was due to the extra time required for the methyl bromide fumigation. Twenty mL of inoculum (for experiment 1), or

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the same amount of PGPR or distilled water (for experiment 2) per one meter of row were applied by syringe directly onto the seeds in the furrow. Cross contamination was prevented throughout planting and all subsequent data collection procedures by alcohol sterilization of all implements used. Following emergence seedlings were thinned to achieve a stand of 500,000 plants ha^{-1} (20 plants m^{-1} of row).

^{15}N application

To be able to measure the seasonal N_2 fixation rates by the isotope dilution method, ^{15}N was applied (1.2 kg ha^{-1}, 99% pure, Isotec Inc., Miamisburg, OH, USA) as double-labelled ammonium nitrate in solution, to a microplot of six plants (30 x 40 cm) within each subplot in the first three replications at both the sterilized and unsterilized sites in both experiments. Each microplot was bordered by plastic sheeting extending 15 cm into the soil to prevent lateral soil losses of the labelled nitrogen. The labelled nitrogen was applied at growth stage V1 (the first unifoliate leaf) (Fehr et al., 1971).

Data collection

One month after planting, the onset of N_2 fixation was tested for. Acetylene reduction activity assays were used as a +/- measure of nitrogenase activity. From each sub-plot four plants were randomly selected, uprooted and detopped; the roots then were exposed to 10% acetylene in a sealed 1L Mason jar for 10 min. A 0.5 mL gas aliquot was then extracted and analyzed by gas chromatography (Hardy et al., 1968). As acetylene reduction activity was detected in all the PGPR application plots, the number, weight, and nitrogen concentration of nodules were measured.

The final nodulation data were taken from plants harvested on August 13, when the plants reached reproductive stage 6 [pod(s) containing a green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed leaf (Fehr et al., 1971)]. The

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plants were uprooted, the roots were washed with distilled water and the nodules were removed, counted and weighed. Microplot materials were harvested by hand at harvest maturity, oven-dried at 70¡C for at least 48 h, and weighed. The seeds were threshed by hand and ground using a Moulinex coffee mill (Moulinex Appliances Inc., Virginia Beach, VA, USA). The above-ground plant tissue from each microplot was ground to pass a 1 mm screen of a Wiley mill (A. H. Thomas Co., Philadephia, PA, USA). The nitrogen concentration of grain and other plant tissues was then determined by Kjeldahl analysis (Kjeltec system, Tecator AB, Hoganas, Sweden). Following Kjeldahl analysis, a sample of the distillate obtained from the shoot and grain material of each microplot was dried and the ammonium present converted to nitrogen gas by the application of the Dumas method (Preston et al., 1981) before measuring the ^{15}N:^{14}N ratio of each sample by emission spectrometry (Jasco N-150 ^{15}N analyzer, Japan Spectroscopic Co., Tokoyo, Japan). The proportion of the total plant nitrogen derived from N_2 fixation was then determined following the formula described by Lynch et al. (1993):

N% from fixation

= {1 - [(^{15}N:^{14}N of fixing plant)/(^{15}N:^{14}N of control plant)]} x 100.

Statistical analysis

The data were analyzed statistically by analysis of variance using the Statistical Analysis System (SAS) computer package (SAS Institute Inc., 1988), except for the onset of nitrogen fixation data (presented in Table 1), which were compared by the Cochran Q test (Hollander et al., 1973). When analysis of variance showed treatment effects ($p \leq 0.05$), the least significant difference (LSD) test was applied to make comparisons among the means ($p \leq 0.05$) (Steel et al., 1980).

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RESULTS

Native Soil *B. japonicum* Levels

At the sterilized site, the fumigation with methyl bromide was not completely effective and the control plants formed adequate nodules at almost the same rate as the inoculated plants. At the unsterilized site, in experiment 2, a small number (less than 3 per plant) of large nodules were formed. Because at least some contamination occurred at both sites, the non-nodulating plants were used as the reference for estimating seasonal N_2 fixation. Because of the higher levels of contamination at the sterilized site, comparisons between strains could not be made with confidence, therefore, only the effects of PGPR application, soybean cultivar and the two way interaction between PGPR application and soybean cultivar were tested.

PGPR effects on nodulation and onset of N_2 fixation

Acetylene reduction activity was used as a +/- indicator of the onset of N_2 fixation in experiment 1 and showed that inoculation with PGPR resulted in a two to four days earlier onset of N_2 fixation on both sites (Table 1).

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TABLE 1

The time of onset of nitrogen fixation by soybean plants inoculated with *B. japonicum* alone or co-inoculated either with PGPR 2-68 or 1-102 at both the unfumigated and fugimated sites. The onset of nitrogen fixation was indicated by detection of acetylene reduction activity.

Site	PGPR	June 13	June 15	June 17	June 21	June 23	June 28
unfumigated	2-68	0/16	2/16	13/16	____	____	____
	1-102	0/16	6/16	16/16	____	____	____
	Control	0/16	1/16	9/16	____	____	____
	Probability	NS	0.05	0.05			
fumigated	2-68	____	____	____	0/12	11/12	12/12
	1-102	____	____	____	0/12	8/12	12/12
	Control	____	____	____	0/12	1/12	9/12
	Probability				NS	0.05	5.05

Values in the table indicate the number of plots in which nitrogen fixation was detected over the total number of plots. Within each site, the data were compared by the Cochran Q test.

B. japonicum USDA110 or 532C co-inoculated with PGPR 2-68 increased both nodule weight per plant and weight per nodule for both AC Bravor and Maple Glen at early soybean growth stages relative to inoculation of the *B. japonicum* strains alone (Table 2). This increase in mass was due to the formation of larger nodules, not to increased nodule number per plant (Table 2). At the sterilized site, Maple Glen plants receiving PGPR 1-102 caused a 100% increase in the individual nodule weight (Table 3). At the second sampling, August 13, Maple Glen plants co-inoculated with *B. japonicum* USDA110 and PGPR 2-68 had increased nodule numbers and nodule weights per plant compared to the plants receiving *B. japonicum* USDA110 alone at the unsterilized site (Table 2).

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TABLE 2

Effects of PGPR strain, *B. japonicum* strain, and soybean cultivar on soybean nodule number, nodule weight, and nodule nitrogen concentration at two harvest stages at the unfumigated site (experiment 1).

			Sampling on June 17					Sampling on August 13		
PGPR	*B. japonicum*	cultivar	Nodule Number	Nodule weight		Nodule nitrogen		Nodule number	Nodule weight (mg)	
				(plant^{-1})	(Nodule^{-1})	(mg g^{-1})	(mg plant^{-1})		(plant^{-1})	(nodule $^{-1}$)
1-102	USDA110	ACBravor	13.92	24.60	2.78	42.81	2.75	84.17	519.40	6.18
		Maple Glen	14.00	97.13	7.94	34.14	3.33	68.67	646.55	9.53
	532C	ACBravor	10.58	42.20	4.40	40.99	1.71	71.00	632.89	9.36
		Maple Glen	15.33	56.82	3.61	39.44	2.29	90.42	804.54	9.61
2-68	USDA110	ACBravor	13.33	78.86	6.00	40.26	3.17	52.92	502.96	10.49
		Maple Glen	13.30	76.27	6.35	40.27	3.08	103.00	923.00	9.01
	532C	ACBravor	11.33	52.18	4.90	44.57	2.32	43.33	537.64	12.60
		Maple Glen	14.92	86.43	6.52	39.15	3.35	90.75	742.23	8.49
Control	USDA110	ACBravor	18.33	11.57	0.62	47.63	0.57	37.83	380.99	10.07
		Maple Glen	19.33	15.73	0.82	32.46	0.51	34.08	410.64	13.03
	532C	ACBravor	10.50	7.27	0.70	46.38	0.34	46.25	427.32	11.67
		Maple Glen	12.33	6.67	0.53	41.09	0.29	60.38	440.39	10.54

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TABLE 2 (cont'd)

Effects of PGPR strain, *B. japonicum* strain, and soybean cultivar on soybean nodule number, nodule weight, and nodule nitrogen concentration at two harvest stages at the unfumigated site (experiment 1).

			Sampling on June 17					Sampling on August 13		
PGPR	*B. japonicum*	cultivar	Nodule Number	Nodule weight		Nodule nitrogen		Nodule number	Nodule weight (mg)	
				($plant^{-1}$)	($Nodule^{-1}$)	($mg\ g^{-1}$)	($mg\ plant^{-1}$)		($plant^{-1}$)	($nodule^{-1}$)
$LSD_{0.05a}$			8.29	19.59	2.78	10.86	1.79	33.32	274.11	4.83
$LSD_{0.05b}$			9.29	20.33	3.33	10.25	1.64	35.86	321.77	6.80
PGPR			NS	***	**	NS	***	**	*	NS
	B. japonicum		*	*	NS	NS	NS	NS	NS	NS
		cultivar	NS	***	*	***	NS	**	***	NS
PGPR	*B.japonicum*		NS	NS	NS	NS	NS	NS	NS	NS
PGPR		*cultivar	NS	**	NS	NS	NS	**	NS	NS
PGPR	*B.japonicum	*cultivar	NS	***	**	NS	NS	NS	NS	NS

Values represent the five plants in the ^{15}N microplot (area equal to 0.12 m^2) from each subplot unit. Means within the same column were compared by an ANOVA protected LSD test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the main plot factor. NS, *, ** and *** indicated no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

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TABLE 3

Simple effect means for PGPR strain and soybean cultivar effects on soybean nodule number, nodule weight, and nodule nitrogen concentration at two harvest stages at the fumigated site (experiment 1).

		Sampling on June 17					Sampling on August 13		
PGPR	cultivar	Nodule Number	Nodule weight		Nodule nitrogen		Nodule number	Nodule weight (mg)	
			(plant^{-1})	(Nodule^{-1})	(mg g^{-1})	(mg plant^{-1})		(plant^{-1})	(nodule $^{-1}$)
1-102	AC Bravor	11.90	21.69	1.78	34.56	0.79	57.58	180.13	3.35
	Maple Glen	14.17	29.17	2.26	33.20	0.95	78.17	211.59	2.66
2-68	AC Bravor	16.08	22.27	1.21	28.82	0.67	55.08	113.99	2.17
	Maple Glen	21.25	33.78	1.66	35.78	1.19	61.00	108.04	1.90
Control	AC Bravor	14.10	15.23	1.18	28.48	0.44	43.00	97.11	2.19
	Maple Glen	20.75	21.18	1.03	32.53	0.69	59.50	125.93	2.14
$LSD_{0.05a}$		14.33	18.62	0.94	8.93	0.61	21.33	73.77	1.05
$LSD_{0.05b}$		12.09	20.79	0.98	11.34	0.58	25.83	77.14	1.47

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TABLE 3 (cont'd)

Simple effect means for PGPR strain and soybean cultivar effects on soybean nodule number, nodule weight, and nodule nitrogen concentration at two harvest stages at the fumigated site (experiment 1).

		Sampling on June 17				Sampling on August 13			
PGPR	cultivar	Nodule Number	Nodule weight		Nodule nitrogen		Nodule number	Nodule weight (mg)	
			(plant^{-1})	(Nodule^{-1})	(mg g^{-1})	(mg plant^{-1})		(plant^{-1})	(nodule $^{-1}$)
PGPR		NS	NS	*	NS	NS	*	**	NS
cultivar		NS	**	NS	NS	***	***	NS	*
PGPR*cultivar		NS	NS	NS	NS	NS	NS	NS	NS

Values represent the five plants in the ^{15}N microplot (area equal to 0.12 m^2) of each subplot unit. Means within the same column were compared by an ANOVA protected LSD test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the main plot factor. NS, *, **, and *** indicate no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

Effects of PGPR on seasonal N_2 fixation

AC Bravor plants co-inoculated with *B. japonicum* USDA110 and PGPR 1-102 increased seasonal N_2 fixation by 100% as compared to plants inoculated with *B. japonicum* USDA110 alone by the N difference method and 92% by the ^{15}N dilution method at the unsterilized site (Table 4), however, there was no increase in the seasonal N_2 fixation for the same treatment combination at the sterilized site (Table 5). Co-inoculation with *B. japonicum* USDA110 and PGPR 2-68 increased seasonal N_2 fixation by 100% relative to *B. japonicum* USDA110 alone for AC Bravor plants by the N difference method at both the sterilized and unsterilized sites (Table 4 and 5). The same combination increased the seasonal N_2 fixation 94% by the ^{15}N dilution method at both the unsterilized and sterilized sites (Tables 4 and 5). Co-inoculation with *B. japonicum* USDA110 and PGPR 1-102 increased the total plant nitrogen yield of AC Bravor plants by 63% relative to *B. japonicum* USDA110 alone at the unsterilized site (Table 4) but caused no increase at the sterilized site (Table 5). Co-inoculation with *B. japonicum* USDA110 and PGPR 2-68 increased total plant nitrogen yield of AC Bravor plants by 50% relative to *B. japonicum* USDA110 alone at the unsterilized site (Table 4) and by 31% increase at the sterilized site (Table 5).

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TABLE 4

Simple effects means for PGPR strain, *B. japonicum* strain, and soybean cultivar for fixed nitrogen, nitrogen concentration and yield at final harvest at the unfumigated site (experiment 1).

			N-difference		^{15}N dilution			
PGPR	*B. japonicum*	cultivar	Total fixed N (kg ha^{-1})	Fixed N as % of total plant N	Total fixed N (kg ha^{-1})	Fixed N as % of total plant N	N Concentration (mg g^{-1})	N yield (kg ha^{-1})
1-102	USDA110	ACBravor	249.96	56.88	113.96	26.44	25.20	417.39
		Maple Glen	166.04	49.50	105.66	31.48	30.93	333.98
	532C	ACBravor	87.30	34.09	80.98	31.25	28.84	255.23
		Maple Glen	79.95	33.26	76.66	31.29	31.79	247.88
2-68	USDA110	ACBravor	214.99	53.54	115.08	29.16	26.57	382.92
		Maple Glen	162.37	47.81	121.74	37.34	24.41	330.31
	532C	ACBravor	152.24	48.11	92.24	28.76	25.28	323.08
		Maple Glen	114.85	39.09	86.75	30.36	29.26	282.79
Control	USDA110	ACBravor	87.76	34.40	59.32	23.69	27.93	255.69
		Maple Glen	127.58	42.58	81.38	27.75	24.82	295.52
	532C	ACBravor	123.64	40.51	69.87	23.87	28.81	288.26
		Maple Glen	41.54	19.2	57.66	27.60	28.01	209.48

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TABLE 4 (cont'd)

Simple effects means for PGPR strain, *B. japonicum* strain, and soybean cultivar for fixed nitrogen, nitrogen concentration and yield at final harvest at the unfumigated site (experiment 1).

			N-difference		^{15}N dilution			
PGPR	*B. japonicum*	cultivar	Total fixed N (kg ha^{-1})	Fixed N as % of total plant N	Total fixed N (kg ha^{-1})	Fixed N as % of total plant N	N Concentration (mg g^{-1})	N yield (kg ha^{-1})
$LSD_{0.05a}$			53.90	8.89	23.38	5.53	4.25	51.22
$LSD_{0.05b}$			49.73	8.99	22.32	5.10	4.30	49.65
PGPR			***	***	***	***	NS	***
	B. japonicum		***	***	***	NS	***	***
		cultivar	***	***	NS	***	*	***
PGPR	*B.japonicum*		***	**	*	*	NS	***
PGPR		*cultivar	NS	NS	NS	NS	***	NS
PGPR	*B.japonicum	*cultivar	***	***	NS	NS	*	***

Values represent the five plants in the ^{15}N microplot (area equal to 0.12 m^2) from each subplot unit. Means within the same column and site were analyzed by an ANOVA protected LSD test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the main plot factor. NS, *, ** and *** indicated no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

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TABLE 5

Simple effects means of PGPR strain and soybean cultivar on fixed nitrogen, nitrogen concentration and yield at final harvest at the fumigated site (experiment 1).

		N-difference		^{15}N dilution			
PGPR	cultivar	Total fixed N	Fixed N as % of	Total fixed N	Fixed N as % of	N Concentration	N Yield
		(kg ha^{-1})	total plant N	(kg ha^{-1})	total plant N	(mg g^{-1})	(kg ha^{-1})
1-102	AC Bravor	100.30	38.14	59.21	21.90	24.23	268.33
	Maple Glen	36.60	19.03	38.87	18.89	28.05	198.65
2-68	AC Bravor	143.15	44.63	88.14	28.06	27.23	311.08
	Maple Glen	86.38	33.66	47.35	19.89	25.09	250.89
Control	AC Bravor	68.98	26.68	46.80	20.33	26.18	236.91
	Maple Glen	66.25	26.58	48.88	22.25	26.13	236.69
$LSD_{0.05a}$		53.94	14.59	18.26	8.57	6.19	59.27
$LSD_{0.05b}$		47.35	12.54	18.06	8.00	4.97	52.53

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TABLE 5 (cont'd)

Simple effects means of PGPR strain and soybean cultivar on fixed nitrogen, nitrogen concentration and yield at final harvest at the fumigated site (experiment 1).

		N-difference		^{15}N dilution			
PGPR	cultivar	Total fixed N	Fixed N as % of	Total fixed N	Fixed N as % of	N Concentration	N Yield
		(kg ha^{-1})	total plant N	(kg ha^{-1})	total plant N	(mg g^{-1})	(kg ha^{-1})
PGPR		**	**	**	NS	NS	**
cultivar		**	**	***	*	NS	***
PGPR*cultivar		NS	NS	***	*	NS	NS

Values represent the five plants in the ^{15}N microplot (area equal to 0.12 m^2) of each subplot unit. Means within the same column were compared by an ANOVA protected LSD test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the main plot factor. NS, *, **, and *** indicate no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

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Co-inoculation with *B. japonicum* USDA110 and PGPR 1-102 increased seasonal N_2 fixation of Maple Glen plants 30% as estimated by the ^{15}N dilution method when compared to the *B. japonicum* USDA110 alone at the unsterilized site (Table 4), however, there was no increase in the seasonal N_2 fixation for the same treatment combination at the sterilized site (Table 5). Maple Glen plants co-inoculated with *B. japonicum* USDA110 and PGPR 2-68 increased seasonal N_2 fixation levels, estimated by the ^{15}N dilution method, 50% higher than those of plants inoculated with USDA110 alone at the unsterilized site (Table 4). The same combination did not increase the seasonal N_2 fixation at the sterilized site (Table 5). Interactions between PGPR application, *B. japonicum* strains, and soybean cultivars existed for total fixed N and fixed N as a percentage of total plant N indicating that 532C and USDA110 had different sensitivities to PGPR preincubation at the unsterilized site (Table 4). Interactions between PGPR application and soybean cultivars indicated that AC Bravor tended to be more responsive to both PGPR treatments for total fixed N, fixed N as a percentage of total plant N, and N yield in the unsterilized site (Table 5).

PGPR effects on soybean nodulation and N fixation through native *B. japonicum*

In experiment 2, where plots were not inoculated with *B. japonicum*, application of PGPR onto seeds in the furrow increased the number of nodules at the sterilized site at the first sampling date (Table 6). At crop maturity, nodule number was increased by PGPR at the sterilized site. PGPR application, in the absence of *B. japonicum* inoculation, also increased seasonal N_2 fixation at both the unsterilized and sterilized sites (Table 7). The final total nitrogen yield of plants receiving PGPR 2-68 was 57% greater than that if plants receiving only distilled water at the unsterilized site.

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TABLE 6

Main effects of PGPR applied directly onto seed in furrow at the time of planting at both fumigated and unfumigated sites (experiment 2).

Site	PGPR	First sampling* Nodule number	First sampling* Nodule weight (mg) (plant^{-1})	First sampling* Nodule weight (mg) (nodule^{-1})	Sampling on August 13 Nodule number	Sampling on August 13 Nodule weight (mg) (plant^{-1})	Sampling on August 13 Nodule weight (mg) (nodule^{-1})
unfumigated site	1-102	3.38	13.00	3.78	20.83	262.50	13.21
	2-68	3.30	14.38	4.56	19.92	250.54	13.97
	Control	3.80	18.00	4.63	22.70	286.42	12.22
	Probability	NS	NS	NS	NS	NS	NS
fumigated site	1-102	9.33	16.86	1.79	18.46	52.44	2.89
	2-68	10.50	12.00	1.35	15.13	48.40	3.10
	control	4.30	9.28	1.94	14.50	36.44	2.68
	Probability	0.05	NS	NS	0.05	NS	NS

* The date of first sampling was the same as experiment 1 for the unfumigated site, June 17, for the sterilized was June 28. Means within the same column and site were analyzed by an ANOVA protected t test.

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TABLE 7

Effects of PGPR applied directly onto soybean seeds in the furrow at the time of planting on fixed nitrogen tissue, nitrogen concentration and yield at final harvest at both the unfumigated and fumigated sites (experiment 2).

Treatment	N-difference		^{15}N dilution			
	Total fixed N	Fixed N as % of	Total fixed N	Fixed N as % of	N Concentration	N yield
	(kg ha^{-1})	total plant N	(kg ha^{-1})	total plant N	(mg g^{-1})	(kg ha^{-1})
unfumigated site						
1-102	49.76	22.62	64.90	29.82	22.03	217.69
2-68	114.79	40.00	83.45	31.03	21.41	278.54
Control	9.17	4.92	36.64	21.34	23.16	177.10
Probability	0.05	0.05	0.05	0.05	NS	0.05
fumigated site						
1-102	77.86	27.06	44.38	19.19	25.47	241.85
2-68	49.00	20.88	41.40	19.37	25.22	214.40
Control	32.83	19.00	36.06	18.47	17.67	205.27
Probability	0.05	0.05	NS	NS	0.05	0.05

Values represent the five plants in the ^{15}N microplot (area equal to 0.12 m^2) from each subplot unit. Means within the same column and site were analyzed by an ANOVA protected t test.

DISCUSSION

The two PGPR strains *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 used in this study were selected based on work reported by Zhang et al., (see Example 3 and 1996b), in which nine PGPR strains were tested for effects on soybean nodulation and nitrogen fixation over a range of RZTs under controlled environment conditions. In our field study, the onset of nitrogen fixation by plants receiving bradyrhizobia preincubated with PGPR was two to three days earlier than those receiving no PGPR treatment at both the unsterilized and sterilized sites (Table 1), while PGPR increased the number of nodules per plant at the unsterilized site. Application of PGPR directly onto the seeds in the furrow at the time of planting also improved plant nodulation and N_2 fixation. The findings of this field study agreed with results from controlled environment work, in which co-inoculation of some PGPR with *B. japonicum* reduced the negative effects of low RZT on soybean nodulation and nitrogen fixation (Zhang et al., see Example 3). In addition, a recent work has shown that inoculation of soybean with PGPRs in the presence and absence of *B. japonicum* increased soybean grain yield, grain protein yield, and total plant protein production under short season areas (unpublished data).

Since nodule dry weight per plant was increased and the onset of nitrogen fixation was hastened by *B. japonicum* co-inoculation with PGPR, total fixed nitrogen and nitrogen yield per plant were increased (Table 5). Sprent (1979) postulated that an increase of ten percent in the period of nodule activity by a grain legumes, particularly between the onset of N_2 fixation and the attainment of maximum fixation, could double the seasonal level of nitrogen fixed. In our experiment, the period of nodule function was about 70 days (late-June to early-September). PGPR application resulted in a two to four day increase in the duration of N_2 fixation (Table 1). PGPR application increased the total amount of nitrogen fixed. It seems likely that

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some of this increase in total fixed nitrogen was due to earlier nitrogen fixation, hastened by PGPR application under short season conditions where soils were stressfully cool in the early growing season, with the remainder being due to increased nodules mass.

The mechanisms of growth and nitrogen fixation promotion by PGPR are not well understood; however, a wide range of possibilities have been postulated, including both direct and indirect actions. Direct actions include an increase in mobilization of insoluble nutrients and subsequent enhancement of uptake by the plants (Lifshitz et al., 1987), production of antibiotics toxic to soil-born pathogens (Li et al., 1988), and production of plant growth regulators that stimulate plant growth (Gaskins et al., 1985). Indirect actions include positive effects on symbiotic nitrogen fixation through enhanced root nodule number or mass (Grimes et al., 1984; Yahalom et al., 1987; Zhang et al., see Example 3) and increased nitrogenase activity (Iruthayathas et al., 1983; Alagawadi et al., 1988).

The results of experiment 2, which investigated the effects of PGPR application without *B. japonicum* addition on soybean growth and development, were different from those observed in experiment 1. At the unsterilized site, although both PGPRs did not increase plant nodule number, they both increased weight per nodule, total fixed nitrogen and N yield (Tables 6 and 7). At the sterilized site, both *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 increased nodule number, total nitrogen fixed, and N yield.

The average proportional increases in nodule number per plant, nodule weight per plant, total fixed nitrogen, and N yield were generally larger in experiment 1 than in experiment 2. There are two possible explanations for this observation. First, in experiment 1, PGPR were perincubated with *B. japonicum* for a period of at least 30 minutes before inoculation, during which PGPR-*B. japonicum* interactions might have

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occurred. Plant growth promoting rhizobacteria produce many phytohormones and signal molecules (Burr et al., 1984; Davison, 1988; Kapulnik, 1991), such as genistein, the plant-to-bacteria signal involved in the soybean nodule infection and formation processes. Therefore, inoculation of soybean plants with *B. japonicum* and PGPR could have resulted in higher relative increases in nitrogen fixation and subsequent soybean growth and yield than *B. japonicum* or PGPR alone. Second, the PGPR may have stimulated overall plant growth, leading to greater nitrogen demand by the developing soybean plants, leading in turn to greater nodulation and nitrogen fixation. The data of Zhang et al. (1996b), showing improved soybean photosynthesis and growth due to PGPR prior to the onset of nitrogen fixation, argue against the former of these two possibilities.

The cultivar AC Bravor tended to be more responsive to inoculation with PGPR plus *B. japonicum* than Maple Glen at the sterilized site (Tables 3 and 5). AC Bravor is a later-maturing cultivar and has a higher potential yield than Maple Glen (Conseil Des Productions Végétales du Québec recommendations); however, at crop physiological maturity it had lower nodule numbers, nodule weight per plant and total nitrogen yield than Maple Glen at the sterilized site (Tables 3 and 5). Therefore, there was less likely to be a nitrogen limitation to the growth and development of Maple Glen than AC Bravor. The increased nodule number and dry matter per plant of AC Bravor due to PGPR application would have reduced any nitrogen limitation; therefore, the increases in total fixed nitrogen and nitrogen yield were greater than those of Maple Glen. At the unsterilized site, the same pattern was found for nodule number and nodule weight per plant for PGPR 1-102 (Table 2). PGPR 2-68 showed a different pattern as it increased nodule number and nodule weight per plant for Maple Glen plants. An interaction also existed between PGPR strain and *B. japonicum* strain (Tables 2 and 4). The combination of USDA110 with PGPR 2-68 or 1-102

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resulted in greater increases in total fixed nitrogen, fixed nitrogen as a percentage of total plant nitrogen, and nitrogen yield than was the case for strain 532C (Table 4).

In the second experiment, plots were not inoculated with *B. japonicum*, and inoculation with PGPR did not increase nodule number at the unsterilized site. However, nodule number increased in the early growing season and at the final harvest at the sterilized site (Table 6). This resulted in an increase in the total fixed nitrogen, tissue N concentration, and N yield (Table 7). It seems that the PGPRs were able to stimulate the native soil *B. japonicum*, resulting in increased soybean nodulation and N_2 fixation in the absence of other soil microflora which might have interfered with this effect.

In summary, this is the first field experiment showing that co-inoculation of *B. japonicum* with PGPR increased soybean nodulation and nitrogen fixation. PGPR application increased nodule dry matter per plant and hastened the onset of nitrogen fixation, especially for early-planted soybean (unsterilized site). Total fixed nitrogen, fixed nitrogen as a percentage of total plant nitrogen, and total nitrogen yield were all increased in at least some cases due to PGPR application. Interactions existed between PGPR application and soybean cultivar indicating that PGPR applied to potentially more nitrogen-stressed plants was more effective. Overall, from this study it was clear that preincubation of *B. japonicum* with PGPR can increase soybean nodulation and nitrogen fixation in the early part of the growing season when soil temperatures are low.

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EXAMPLE 2

Application of plant growth-promoting rhizobacteria to soybean (*Glycine max* [L.] Merr.) increases protein and dry matter yield under short season conditions

We previously reported that application of plant growth-promoting rhizobacteria (PGPR) increased soybean growth and development and, specifically, increased nodulation and nitrogen fixation over a range of root zone temperatures (RZTs) in controlled environment studies. In order to expand on the previous studies, field experiments were conducted on two adjacent sites, one fumigated with methyl bromide and one nonfumigated, in 1994. Two experiments were conducted at each site, one involving combinations of two soybean cultivars and two PGPR strains, the other involving the same factors, but also in combination with two strains *Bradyrhizobium japonicum*. Soybean grain yield and protein yield were measured. The results of these experiments indicated that co-inoculation of soybean with *B. japonicum* and *Serratia liquefaciens* 2-68 or *Serratia proteamaculans* 1-102 increased soybean grain yield, protein yield, and total plant protein production, compared to the nontreated controls, in an area with low spring soil temperatures. Interactions existed between PGPR application and soybean cultivar, suggesting that PGPRs applied to cultivars with higher yield potentials were more effective. PGPRs applied into the rhizosphere without addition of *B. japonicum* also increased plant grain yield 22%, protein yield 13% and total plant protein 23%. Overall, inoculation of soybean plants with PGPRs in the presence and absence of *B. japonicum* increased soybean grain yield, grain protein yield, and total plant protein production under short season conditions.

MATERIALS AND METHODS

Field layout and site preparation

Two experiments were conducted at the Emile A. Lods Research Centre, McGill University, Macdonald Campus, Montreal, Canada. They were performed at two adjacent sites. One site was fumigated with methyl bromide (50 g m^{-2}) applied under a plastic canopy for 72 hours, to prevent possible interference from soil microfloral or faunal elements that might obscure PGPR treatment effects. Three days elapsed between removal of the fumigation canopy and planting. The soil of the other site was left nonfumigated.

The experimental design was a 3 x 2 x 2 factorial organized in a randomized complete block split-plot with four replications. The first experiment included three factors, PGPR application, *B. japonicum* strain, and soybean cultivar. The main-plot units consisted of PGPR strain applications (no-PGPR application as a control, *Serratia liquefaciens* 2-68 and *Serratia proteamaculans* 1-102). The two strains tested were chosen based on the results of a previous controlled environment experiment (Zhang et al., 1996c). The subplot units were formed by the combination of soybean cultivars and *B. japonicum* strains. The soybean cultivars, Maple Glen and AC Bravor were selected as they have been developed for production under the short season, cool conditions of eastern Canada. Both are widely grown and yield well in this area. The *B. japonicum* strains tested were 532C (Hume et al., 1990) and USDA110, both of which are or have been included in commercial inoculants used in eastern Canada. In the second experiment two factors were tested, PGPR strains, and soybean cultivars. The design of the experiment was the same as experiment 1. Three levels of PGPR applications (no-PGPR control, *S. liquefaciens* 2-68, and *S. proteamaculans* 1-102) formed the main plot units, and two soybean cultivars (Maple Glen and AC Bravor) were the subplot units. At the nonfumigated site, each

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sub-plot (2 x 3 m) consisted of four rows of plants with 40 cm between rows. The space between plots was 80 cm and between replications 1 m. At the fumigated site, each sub-plot was 1.6 x 2 m and consisted of three rows of plants, also with 40 cm between rows. The space between plots was 80 cm and between replications, 2 m. The soil type at both sites was a Chicot light sandy loam. In the previous year, 1993, this experimental field was planted with oat and barley, while in 1992 it was used to produce a crop of green manure alfalfa. The soil nitrogen available for soybean uptake proved to be reasonably high. The average nitrogen accumulation in non-nodulating soybean plants seeded at the same site was 167 kg ha^{-1}. Potassium and phosphate were provided by spring application of 340 kg ha^{-1} of 5-20-20, N, P_2O_5, K_2O, according to a soil test.

Inoculum preparation

For experiment 1, the inoculum was produced by culturing *B. japonicum* strains 532C and USDA110 in yeast extract mannitol broth (Vincent, 1970) in 2000 mL flasks shaken at 125 rpm at room temperature (23-25°C). The PGPR strains were cultured in *Pseudomonas* media (Polonenko et al. 1987) in 2000 mL flasks shaken at 250 rpm at room temperature (21-23°C). After both *B. japonicum* and PGPR reached the stationary phase (7-days for *B. japonicum* and 1.5-days for PGPR), they were subcultured under the same conditions as described above. When the subculture reached the log phase (3-days for *B. japonicum* and 1-day for PGPR), *B. japonicum* and PGPR strains were each adjusted with distilled water to an A_{620} and A_{420} value, respectively, giving a cell density of 2×10^8 cells mL^{-1}. Before inoculation equal volumes of *B. japonicum* and PGPR cultures were mixed and allowed to stand for a minimum of 30 min at room temperature.

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Planting methods

Seed of the soybean cultivars 'Maple Glen' and 'AC Bravor' were surface-sterilized in sodium hypochlorite (2% solution containing 4 mL L^{-1} Tween 20), then rinsed several times with distilled water (Bhuvaneswari et al., 1980). The seeds were planted by hand on May 11 and 18 at the nonfumigated and fumigated sites, respectively. The delay in planting the fumigated site was due to the extra time required for the methyl bromide application. Twenty mL of combined *B. japonicum*-PGPR inoculum (for experiment 1), or the same volume and cell density of PGPR inoculum or the same volume of distilled water (for experiment 2) per one meter row were applied by syringe directly onto the seed in the furrow. Cross contamination was prevented throughout planting and all subsequent data collection procedures by alcohol sterilization of all implements used. Following emergence seedlings were thinned to achieve a stand of 500,000 plants ha^{-1} (20 plants m^{-1} of row).

Data collection

Plant samples were taken on August 13, at which time plants were at reproductive stage 6 (pod containing a green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed leaf [Fehr et al., 1971]), to investigate growth variables, such as leaf number, leaf area, pod number and seed number. Leaf number and area per plant were determined using a Delta-T area meter (Delta-T Devices Ltd., Cambridge, UK). Pod number and seed number per plant were counted by hand. Grain yield was determined from a one meter row of plants taken from the middle row of each plot. Plants were harvested by hand at harvest maturity, then shelled with a plot combine (Wintersteiger, Salt Lake City, UT), oven-dried at 70°C for at least 48 hours, and weighed. Six additional plants, also from the middle row, were hand-harvested, and oven-dried at 70°C, after which seeds were manually separated from shoots. Total shoot weight

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and harvest index were determined from these plants, which had been enclosed within wire mesh from flowering to maturity to allow the collection of senescent leaves. The dried seeds from each plot were ground using a Moulinex coffee mill (Moulinex Appliances Inc., Virginia Beach, VA, USA). The nitrogen concentration of seeds was then determined by Kjeldahl analysis (Kjeltec system, Tecator AB, Hoganas, Sweden). The protein concentration was calculated by multiplying the nitrogen concentration by 6.25.

Statistical analysis

Results were analyzed statistically by analysis of variance using the Statistical Analysis System (SAS) computer package (SAS Institute Inc., 1983). When analysis of variance showed significant treatment effects, the least significant difference (LSD) test was applied to make comparisons among the means at the 0.05 level of significance (Steel et al., 1980).

RESULTS

Temperature and seed emergence

The average daily temperature for both air and soil (at a depth of 5 cm) was below 15°C through early June, and remained below 20°C until mid-July. These conditions slowed the rate of seedling emergence, particularly for the plants at the nonfumigated site (early planted). For the May 11 planting, at the nonfumigated site, seedlings emerged on May 25, 14 days after planting. At the fumigated site, the seeds were planted on May 18 and the seeding emerged at 9 DAP, only 2 days after the nonfumigated site.

Experiment 1

The nodule number of uninoculated plants in experiment 2 indicated that the native soil population of *B. japonicum* in the nonfumigated soil was low, with uninoculated plants forming few nodules. However, at the fumigated site, fumigation with methyl bromide was not completely effective

and the un-inoculated plants in experiment 2 formed nearly as many nodules as the inoculated plants in experiment 1. Therefore neither of the two interactions relating to *B. japonicum* strain nor the three way interaction were tested at the fumigated site.

Many growth variables, such as plant height, time of crop maturity, harvest index, and seed moisture content at harvest maturity, were not affected by the inoculation of PGPR at either site (data not shown). Overall, leaf number was increased by PGPR application. The leaf area of AC Bravor receiving USDA110 was increased by inoculation with a mixture of *B. japonicum* and PGPR at the nonfumigated site (Table 8), while at the fumigated site leaf area was increased by PGPR application across all levels of *B. japonicum* strain and soybean cultivar, except for PGPR 2-68 with Maple Glen (Table 9). Differences between the two PGPR strains were not observed for any plant growth variables at either site.

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TABLE 8

Effects of PGPR application, *B.japonicum* strains, and soybean cultivars on soybean growth variables, grain yield, final protein and grain yield in a nonfumigated field trial (experiment 1).

PGPR	*B. Japonicum*	cultivar	Leaf (plant^{-1}) number	Leaf (plant^{-1}) Area(cm^2)	Number (plant^{-1}) Pod	Number (plant^{-1}) Seed	1000 seeds weight(g)	Yield (t ha^{-1}) Grain	Yield (t ha^{-1}) Grain Protein	Yield (t ha^{-1}) Total protein	Protein Concentration (mg g^{-1})
1-102	USDA110	AC Bravor	31.3	976.2	29.0	69.0	190.33	5.4	2.2	2.7	401.3
		Maple Glen	19.2	518.3	20.2	50.0	190.33	4.3	1.7	2.0	386.5
	532C	ACBravor	27.8	977.1	28.7	68.3	161.00	5.0	1.9	2.1	395.4
		Maple Glen	15.2	425.6	19.6	48.3	190.33	3.1	1.3	1.5	365.0
2-68	USDA110	ACBravor	31.3	830.0	28.3	70.3	194.67	5.4	1.9	2.7	391.5
		Maple Glen	26.3	805.4	31.3	75.0	189.00	4.9	1.9	2.3	416.6
	532C	ACBravor	29.0	1044.2	27.0	45.0	195.00	4.6	1.7	2.1	386.5
		Maple Glen	16.4	628.0	18.3	46.0	169.33	3.3	1.3	1.8	380.5
Control	USDA110	ACBravor	16.0	516.0	16.0	45.7	180.33	4.4	1.8	2.1	373.8
		Maple Glen	21.4	543.0	24.4	57.3	195.67	3.8	1.4	1.8	365.0
	532C	ACBravor	21.1	910.0	26.3	59.0	201.67	4.9	1.8	2.0	380.5
		Maple Glen	14.8	533.0	24.1	35.0	196.00	3.1	1.2	2.1	333.5

TABLE 8 (cont'd)

Effects of PGPR application, *B. japonicum* strains, and soybean cultivars on soybean growth variables, grain yield, final protein and grain yield in a nonfumigated field trial (experiment 1).

PGPR	*B. Japonicum*		cultivar	Leaf (plant^{-1}) number	Leaf (plant^{-1}) Area(cm^2)	Number (plant^{-1}) Pod	Number (plant^{-1}) Seed	1000 seeds weight(g)	Yield (t ha^{-1}) Grain	Yield (t ha^{-1}) Grain Protein	Yield (t ha^{-1}) Total protein	Protein Concentration (mg g^{-1})
LSD_a				7.6	240.4	7.0	15.4	36.20	0.5	0.3	0.3	32.4
LSD_b				6.9	239.5	6.7	16.3	35.14	0.6	0.4	0.3	30.0
PGPR				***	**	**	***	NS	***	***	***	***
B.japonicum				**	NS	NS	***	NS	***	***	***	**
cultivar				***	***	NS	NS	NS	***	***	***	**
PGPR x *B.japonicum*	NS	NS			***	**	NS	***	***	***	NS	
PGPR x cultivar	**	**			*	NS	NS	**	***	***	*	
PGPR x *B.japon-icum* x cultivar	NS	NS			NS	***	NS	NS	***	***	NS	

Means of leaf number and leaf area and seed number represent four plants from each subplot unit, at crop maturity. Means of 1000 seed, weight calculated from the one meter middle row of each subplot unit at harvest maturity. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference at the 0.1, 0.05, and 0.01 levels, respectively.

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TABLE 9

Effects of PGPR application and soybean cultivars on soybean growth variables, grain yield, final protein and grain yield in a fumigated field trial (experiment 1).

PGPR	Cultivar	Leaf (plant^{-1})		Number (plant^{-1})		1000 seeds weight(g)	Yield (t ha^{-1})			Protein Concentration (mg g^{-1})
		number	Area(cm^2)	Pod	Seed		Grain	Grain Protein	Total protein	
1-102	AC Bravor	52.9	893.3	46.2	80.5	160.5	3.8	1.5	1.8	396.7
	Maple Glen	41.8	473.7	36.0	57.7	152.1	2.1	0.8	1.0	382.6
2-68	ACBravor	56.2	995.8	38.2	73.1	164.7	4.0	1.6	2.0	396.5
	Maple Glen	23.8	239.0	19.8	47.8	155.8	2.3	1.1	1.3	373.8
Control	ACBravor	28.2	472.8	24.7	48.7	154.0	3.1	1.0	1.4	371.2
	Maple Glen	16.0	189.7	18.7	47.8	155.2	3.1	1.0	1.4	365.7
LSD_a		7.1	165.0	8.2	12.6	11.6	0.6	0.3	0.3	15.4
LSD_b		6.0	134.9	9.8	15.4	8.7	0.7	0.3	0.4	12.4
PGPR		***	***	***	**	**	***	***	***	***
cultivar		***	***	***	***	*	***	***	***	***
PGPR x cultivar		***	***	**	***	*	***	***	***	*

Means of leaf number and leaf area and seed number represent four plants from each subplot unit at crop maturity. Means of 1000 seed weight calculated from the one meter middle row of each subplot unit at harvest maturity. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

Averaged over the two PGPR strains, the number of seeds formed on AC Bravor inoculated with a mixture of *B. japonicum* USDA110 and PGPR increased by 52% at the nonfumigated site (Table 8), while at the fumigated site the number of seeds of AC Bravor plants receiving *B. japonicum* and PGPR increased by 58% (Table 9). This increase in seed number was due to increases in their pod numbers (Tables 8 and 9). Since seed number per plant increased and seed protein concentration did not decrease, total grain yield and protein yield also increased. At the nonfumigated site, averaged over the two PGPR strains, the final grain yield of AC Bravor and Maple Glen inoculated with a mixture of *B. japonicum* USDA110 and PGPR increased by 23 and 21%, respectively (Table 8). At the fumigated site the grain yields of AC Bravor receiving *B. japonicum* and either *S. proteamaculans* 1-102 or *S. liquefaciens* 2-68 were 23, and 29% higher than plants receiving only *B. japonicum* (non-PGPR control plants), respectively (Table 9). Again, there was no difference between the two PGPR strains for increase in soybean grain yield.

The main effect of PGPR on protein concentration was significant at both sites. Averaged over all the treatments, the protein concentrations of plants receiving PGPR application were 7.5, and 5.1% higher than those of the non-PGPR control plants at the nonfumigated and fumigated sites, respectively (Tables 8 and 9). Because both final grain yield and protein concentration were increased by inoculating with a mixture of *B. japonicum* and PGPR, the final grain protein and total plant protein yields also increased at both sites. At the nonfumigated site, the grain protein yield of AC Bravor receiving the mixture of USDA110 and *S. proteamaculans* 1-102 was 22% higher than its corresponding control plants (Table 8). At the fumigated site, the protein yield of AC Bravor plants receiving *S. liquefaciens* 2-68, and *S. proteamaculans* 1-102 application increased by 60, and 50% over AC Bravor receiving only *B. japonicum* (Table 9). The total plant protein

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yield increase due to PGPR application generally followed the same pattern as the grain protein yield (Tables 8 and 9).

Two way interactions between either PGPR strain and soybean cultivar, or PGPR strain and *B. japonicum* strain existed for most of the yield related variables investigated in these studies at both sites (Tables 8 and 9). The combination of AC Bravor and *B. japonicum* USDA110 was more sensitive to PGPR application than Maple Glen and 532C.

Experiment 2

Plant growth-promoting rhizobacteria, directly applied onto seeds in the furrow at the time of planting, did not have any effect on growth variables and yield compared to control plants at the nonfumigated site (Table 10) but increased these variables at the fumigated site (Table 11). Generally speaking, at the fumigated site, the effects of PGPR application directly into rhizosphere soil, on soybean growth variables, yield components, and final grain and protein yield followed the same pattern observed in experiment 1. The combination of PGPR 2-68 increased leaf area (36%), seed number (32%), and grain protein yield (20%) of AC Bravor plants. The increase in the grain protein yield was not due to any increase of grain protein concentration.

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TABLE 10

Effects of PGPR application and soybean cultivars on soybean growth variables, grain yield, final protein and grain yield in a nonfumigated field trial (experiment 2).

PGPR	Cultivar	Leaf (plant^{-1}) number	Leaf (plant^{-1}) Area(cm^2)	Number (plant^{-1}) Pod	Number (plant^{-1}) Seed	1000 seeds weight(g)	Yield (t ha^{-1}) Grain	Yield (t ha^{-1}) Grain Protein	Yield (t ha^{-1}) Total protein
1-102	AC Bravor	32.0	754.8	28.3	52.7	177.1	3.2	1.2	1.7
	Maple Glen	23.6	500.8	26.3	73.3	170.9	3.3	1.0	1.4
2-68	ACBravor	30.3	767.8	20.7	49.7	179.11	3.6	1.2	1.4
	Maple Glen	28.7	866.2	21.7	50.7	177.9	3.2	1.0	1.4
Control	ACBravor	23.0	740.4	16.0	35.3	151.3	3.4	1.5	1.7
	Maple Glen	22.7	720.3	15.3	35.0	163.1	3.5	1.0	1.6
LSD_a		15.6	510.2	14.8	29.7	20.0	0.4	0.4	0.4
LSD_b		14.6	445.9	13.9	29.6	18.3	0.4	0.3	0.3
PGPR		NS	NS	NS	NS	NS	NS	NS	NS
cultivar		NS	NS	NS	NS	NS	NS	NS	NS
PGPR x cultivar	NS	NS	NS	NS	NS	NS	NS	NS	

Means of leaf number and leaf area and seed number represent four plants from each subplot unit at crop maturity. Means of 1000 seed weight calculated from the one meter middle row of each subplot unit at harvest maturity. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

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TABLE 11

Effects of PGPR application and soybean cultivars on soybean growth variables, grain yield, final protein and grain yield in a fumigated field trial (experiment 2).

PGPR	Cultivar		Leaf (plant^{-1}) number	Leaf (plant^{-1}) Area(cm^2)	Number (plant^{-1}) Seed	Number (plant^{-1}) Pod	1000 seeds weight(g)	Yield (t ha^{-1}) Grain	Yield (t ha^{-1}) Grain Protein	Yield (t ha^{-1}) Total protein
1-102	Maple Glen		38.0	627.3	82.7	34.0	162.5	2.7	1.1	1.1
	ACBravor		62.0	987.5	86.0	29.5	147.7	3.3	1.3	1.6
2-68	Maple Glen		27.0	338.5	45.7	20.2	151.0	2.6	0.9	1.3
	ACBravor		53.2	756.2	77.7	33.5	151.3	2.7	1.2	1.2
Control	Maple Glen		40.5	849.3	62.2	26.2	155.3	3.0	1.3	1.6
	ACBravor		41.3	683.8	39.6	21.5	148.0	2.5	0.9	1.3
LSD_a			19.3	143.0	13.2	9.1	10.0	0.9	0.3	0.4
LSD_b			17.0	161.6	16.1	8.9	11.0	0.7	0.3	0.3
PGPR			NS	**	***	NS	NS	NS	NS	NS
cultivar			NS	***	NS	NS	NS	NS	NS	NS
PGPR x cultivar		NS	***	***	***	NS	NS	NS	NS	

Means of leaf number and leaf area and seed number represent four plants from each subplot unit at crop maturity. Means of 1000 seed weight calculated from the one meter middle row of each subplot unit at harvest maturity. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

DISCUSSION

Plant growth-promoting rhizobacteria strains *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 were selected following previous controlled environment studies (Zhang et al., see Example 3) in which nine PGPR strains were tested for effects on soybean plant growth, development, nodulation and nitrogen fixation over a range of RZT under controlled environment conditions. Specifically, *S. liquefaciens* 2-68 performed well at optimal RZT (25°C), while *S. proteamaculans* 1-102 performed best at suboptimal RZTs ranging from 18 to 15°C. In our current studies, co-inoculation with PGPR and *B. japonicum* improved plant growth, development, yield components, and final grain and protein yield in the presence and absence of methyl bromide fumigation. Application of PGPR with the *B. japonicum* directly onto the seeds in the furrow at the time of planting also improved plant growth and increased grain and protein yield at the fumigated site. These results agreed with those previously found under controlled environment conditions (Zhang et al., see Example 3). However, the effects of PGPR application on plant growth, development, and final grain and protein yield were not different between *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102. This could be due to variations in field soil temperature during the entire soybean growing season.

Inoculation of soybean plants with a mixture of *B. japonicum* and PGPR not only increased plant dry matter accumulation, but also increased grain protein and total protein production at both sites in experiment 1 (Tables 8 and 9) and at the fumigated site in experiment 2 (Table 11). Zhang et al. (see Example 3) reported that co-inoculation of some PGPR with *B. japoniucm* could reduce the negative effects of low RZT on soybean nodulation and nitrogen fixation. In addition, a recent study at McGill University found that co-inoculation of PGPR and *B. japonicum* accelerated the processes of soybean nodulation and the onset of nitrogen fixation under

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short season field conditions. Sprent (1979) postulated that an increase of 10% in the period of nodule activity of a grain legume, particularly between the onset of nitrogen fixation and the attainment of maximum fixation, could double the seasonal level of nitrogen fixation. In a controlled environment experiment, the onset of nitrogen fixation by plants co-inoculated with *B. japonicum* and the most effective PGPR strains began 2 to 3 days earlier than those receiving only *B. japonicum* (Zhang et al., see Example 3). Therefore, it is possible that application of PGPR increased grain and total protein yield under field conditions.

The mechanisms of growth and nitrogen fixation promotion by PGPR are not well understood; however, a wide range of possibilities have been postulated, including both direct and indirect actions. The direct actions include an increase in mobilization of insoluble nutrients and subsequent enhancement of uptake by the plants (Lifshitz et al., 1987), production of antibiotics toxic to soil-born pathogens (Li et al., 1987), and production of plant growth regulators that stimulate plant growth (Gaskins et al., 1985). Indirect actions include positive effects on symbiotic nitrogen fixation by enhanced root nodule number or mass (Grimes et al., 1984; Yahalom et al., 1987; Zhang et al., see Example 3) and increased nitrogenase activity (Iruthayathas et al., 1983; Alagawadi et al., 1988).

The results of experiment 2, which investigated the effects of PGPR application without *B. japonicum* addition on soybean growth and development, were different from those observed in experiment 1. At the nonfumigated site, although both PGPR *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 numerically increased plant growth variables such as leaf area and seed numbers, there were no statistically significant differences among treatments (Table 10). At the fumigated site, both *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 affected most plant variables, especially on AC Bravor plants. As described above, the native soil population of *B. japonicum*

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in nonfumigated soil was low, with uninoculated plants forming few nodules, whereas at the fumigated site, uninoculated plants formed nearly as many nodules as the inoculated plants in experiment 1. Therefore, the difference in results between the sites may indicate that the PGPR were not able to perform well in the absence of *B. japoniucm* under short season field conditions.

The average proportional increases in plant growth variables, yield components and final grain and protein yield were generally larger in experiment 1 than in experiment 2. There are two possible explanations for this observation. First, as described above, PGPR application may not have acted effectively in the absence of *B. japonicum* under short season field conditions. Second, in experiment 1, PGPR were preincubated *with B. japonicum* for a period of at least 30 minutes before inoculation, during which a number of PGPR-*B. japonicum* interactions might have occurred. Plant growth promoting rhizobacteria produce many phytohormones and signal molecules (Burr et al., 1984; Davison, 1988; Kapulnik, 1991), such as genistein, a plant-to-bacteria signal involved in the soybean nodule infection and formation processes. Preincubation of *B. japonicum* inocula with genistein increased nodule number and hastened the onset of nitrogen fixation at suboptimal RZT under both controlled environment (Zhang et al., 1995c) and short season field (Zhang et al., 1996b) conditions. Therefore, co-inoculation of soybean plants with *B. japonicum* and PGPR could have resulted in higher relative increases in nitrogen fixation and subsequent soybean growth and yield than with *B. japonicum* or PGPR alone.

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EXAMPLE 3

Growth, Survival, and Root Colonization of Plant Growth-Promoting Rhizobacteria under short season conditions

Co-inoculation of *B. japonicum* with plant growth promoting rhizobacteria (PGPR) has been shown to increase soybean [*Glycine max* (L.) Merr.] nodulation, nitrogen fixation, growth, and development compared to controls not inoculated with PGPR, in an area with low spring soil temperatures. We studied the growth and survival of rifampacin resistant strains of two plant growth promoting rhizobacteria (PGPR) *Serratia liquefaciens* 2-68 and *Serratia proteamaculans* 1-102 inoculated on soybean plants under cool spring conditions. Two field experiments were conducted on two adjacent sites, one fumigated with methyl bromide and one not fumigated, in 1994. Two experiments were conducted at each site, one involving combinations of two soybean cultivars, two strains of *Bradyrhizobium japonicum* and two PGPR strains, the other involving the same factors, but without *B. japonicum*. The population density of PGPR applied into the rhizosphere without addition of *B. japonicum* increased over time indicating that the PGPR were able to survive and proliferate. Overall, PGPR inoculated onto soybean roots was able to grow, survive, and colonize the roots better at the fumigated site. PGPR 2-68 achieved higher population densities on both the root and in the soil (rhizosphere) which demonstrates their ability to colonize the root more rapidly.

MATERIALS AND METHODS

Field layout and site preparation

Two experiments at two adjacent sites were conducted at the Emile A. Lods Research Centre, McGill University, Macdonald Campus, Montreal, Canada. One site was fumigated with methyl bromide (50 g m^{-2}) applied under a plastic canopy for 72 hours, to prevent possible interference

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from soil microfloral or faunal elements. Three days elapsed between removal of the fumigation canopy and planting. The soil of the other site was left nonfumigated.

The experiment was arranged in a 3 x 2 x 2 factorial organized in a randomized complete block split-plot with four replications. Experiment 1 included three factors, PGPR application, *B. japonicum* strain, and soybean cultivar. The main-plot units consisted of PGPR strain applications (*Serratia liquefaciens* 2-68 and *Serratia proteamaculans* 1-102). The two strains tested were chosen based on the results of a previous controlled environment experiment (Zhang et al., 1996b). The subplot units were formed by the combination of soybean cultivars and *B. japonicum* strains. The soybean cultivars, Maple Glen and AC Bravor were selected as they have been developed for production under the short season, cool conditions of eastern Canada. Both are widely grown and yield well in this area. The *B. japonicum* strains tested were 532C (Hume and Shelp, 1990) and USDA110, both of which are or have been included in commercial inoculants used in eastern Canada. In experiment 2, only two factors were tested, PGPR strains, and soybean cultivars. The design of experiment 2 was the same as experiment 1. Two levels of PGPR (*S. liquefaciens* 2-68, and *S. proteamaculans* 1-102) formed the main plot units, and two soybean cultivars (Maple Glen and AC Bravor) were the subplot units.

Inoculum preparation

For experiment 1, the inoculum was produced by culturing *B. japonicum* strains 532C and USDA110 in yeast extract mannitol broth (Vincent, 1970) in 2000 mL flasks shaken at 125 rpm at room temperature (23-25°C). Two rifampacin resistant PGPR strains (1-102 *Serratia proteamaculans* and 2-68 *Serratia liquefaciens)* were tested in this experiment. The PGPR strains were cultured in *Pseudomonas* media (Polonenko et al. 1987) in 2000 mL flasks shaken at 250 rpm at room

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temperature (21-23°C). After both *B. japonicum* and PGPR reached the stationary phase (7-days for *B. japonicum* and 1.5-days for PGPR), they were subcultured under the same conditions as described above. When the subculture reached the log phase (3-days for *B. japonicum* and 1-day for PGPR), *B. japonicum* and PGPR strains were each adjusted with distilled water to an A_{620} and A_{420} value, respectively, giving a cell density of 2×10^8 cells mL^{-1}. Before inoculation equal volumes of *B. japonicum* and PGPR cultures were mixed and allowed to stand for a minimum of 30 min at room temperature.

Planting methods

Seed of the soybean cultivars 'Maple Glen' and 'AC Bravor' were surface-sterilized in sodium hypochlorite (2% solution containing 4 mL L^{-1} Tween 20), then rinsed several times with distilled water (Bhuvaneswari et al., 1980). The seeds were planted by hand on May 11 and 18 at the nonfumigated and fumigated sites, respectively. The delay in planting the fumigated site was due to the extra time required for the methyl bromide application. Twenty mL of combined *B. japonicum*-PGPR inoculum (for experiment 1), or the same volume and cell density of PGPR inoculum (for the treatment) or the same volume of distilled water (for the control) (for experiment 2) were applied by syringe directly onto the seed in the furrow. Cross contamination was prevented throughout planting and all subsequent data collection procedures by alcohol sterilization of all implements used.

Enumeration of PGPR

Root and soil samples were collected twice during the experiment. The first sample was collected June 20 when the plants were beginning to bloom [one open flower at any node on the main stem(R1)]. The second sample was collected on August 13 when the plants had reached reproductive stage 6 [pod(s) containing a green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed

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leaf] (Fehr et al., 1971). Number of culturable PGPR cells in the bulk soil (rhizosphere) was determined by transferring 10 g of the soil into a 250-mL Erlenmeyer flask, containing 90 mL of sterile distilled water. The flasks were shaken for 30 min at room temperature (200 rpm). Serial 10-fold dilutions were made and plated on King's B agar (proteose peptone, 20 g; K_2HPO_4, 1.5 g; $MgSO_4$ $7H_2O$, 1.5 g; glycerol, 10 g; agar, 15 g; demineriliized water, 1L; pH 7.2) supplemented with 100 mg L^{-1} cycloheximide (to inhibit fungal growth) and 100 mg L^{-1} rifampacin. Plant roots and adhering soil were seperated from the bulk soil (rhizosphere) by careful, manual shaking. Roots and adhering soil were then transferred into a 250-mL Erlenmeyer flask, containing 90 mL of sterile distilled water. Shaking, dilution and plating procedures were similar to those descibed for the bulk soil (rhizosphere). The plates were incubated for 24 hr and the cfu g^{-1} of dry soil and root were calculated.

Statistical analysis

Results were analyzed statistically by analysis of variance using the Statistical Analysis System (SAS) computer package (SAS Institute Inc., 1983). When analysis of variance showed significant treatment effects, the least significant difference (LSD) test was applied to make comparisons among the means at the 0.05 level of significance (Steel et al.,1980).

RESULTS

Temperature and seed emergence

The average daily temperature for both air and soil (at a depth of 5 10 cm) was below 15°C through early June, and remained around 20°C until mid-July. By August, the temperature start to drop until it reached 10°C by the end of September (Zhang t al., 1995a).

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Experiment 1

The microbial densities associated with the root and in the soil (rhizosphere) of the soybean plants are presented in table 12. The data indicates differences among the PGPR strains. PGPR 2-68 showed more cfu g^{-1} root in the first sampling than PGPR 1-102, while there was no significant difference between the two PGPR at the second sampling at the nonfumigated site. PGPR 2-68, which had higher population densities on the root, also showed higher microbial activities in the soil (rhizosphere) than PGPR 1-102. There was no difference between the two PGPRs in the cfu g^{-1} root or cfu g^{-1} soil at the second sampling of the nonfumigated site (Table 12).

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TABLE 12

The colony forming unit (log cfu gm dry root^{-1} and log cfu gm dry soil^{-1}) values for PGPR strains, in association with *B. japonicum* strains, and soybean cultivars from the nonfumigated field trial (experiment 1).

			(log cfu gm dry root^{-1})		(log cfu gm dry soil^{-1})	
PGPR	*B. japonicum*	Cultivar	June 20	Aug. 13	June 20	Aug. 13
1-102	USDA110	AC Bravor	6.08	5.83	4.02	3.34
		Maple Glen	5.77	5.50	3.94	3.08
	532C	ACBravor	5.66	5.25	4.18	2.98
		Maple Glen	5.59	5.24	3.71	3.82
2-68	USDA110	ACBravor	6.70	7.16	4.52	4.76
		Maple Glen	6.58	6.15	4.66	2.96
	532C	AC Bravor	6.20	6.30	4.76	2.97
		Maple Glen	6.44	6.80	4.68	3.31
LSD_a			0.49	1.0	0.2	0.67
LSD_b			0.69	1.8	0.3	0.62

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TABLE 12 (cont'd)

The colony forming unit (log cfu gm dry root^{-1} and log cfu gm dry soil^{-1}) values for PGPR strains, in association with *B. japonicum* strains, and soybean cultivars from the nonfumigated field trial (experiment 1).

			(log cfu gm dry root^{-1})		(log cfu gm dry soil^{-1})	
PGPR	*B. japonicum*	Cultivar	June 20	Aug. 13	June 20	Aug. 13
PGPR			*	NS	**	NS
B. japonicum			**	NS	**	*
cultivar			NS	NS	NS	NS
PGPR **B.japonicum*			NS	NS	***	**
PGPR*cultivar			NS	NS	*	***
PGPR**B.japonicum**cultivar			NS	NS	**	***

Means within the same column were analyzed by an ANOVA protected t test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot (PGPR strains) unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

At the fumigated site, the same general pattern was observed as PGPR 2-68 showed higher cfu g^{-1} root in both the first and second sampling than PGPR 1-102 which indicate that this strain was able to multiply and colonize the plant roots more effectiently than PGPR 1-102. There was no difference between the two PGPR for the number of cfu g^{-1} root or cfu g^{-1} soil at the second sampling (Table 13). There was no difference observed between the two soybean cultivars, AC Bravor and Maple Glen, at both fumigated and nonfumigated sites (Tables 12 and 13).

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TABLE 13

The colony forming unit (log cfu gm dry root^{-1} and log cfu gm dry soil^{-1}) values for PGPR strains, in association with *B. japonicum* strains, and soybean cultivars from the fumigated field trial (experiment 1).

			(log cfu gm dry root^{-1})		(log cfu gm dry soil^{-1})	
PGPR	*B. japonicum*	Cultivar	June 20	Aug. 13	June 20	Aug. 13
1-102	USDA110	AC Bravor	5.78	6.94	3.24	4.91
		Maple Glen	5.61	6.57	3.34	5.09
	532C	ACBravor	5.03	6.28	3.56	3.88
		Maple Glen	5.50	7.30	3.37	4.55
2-68	USDA110	ACBravor	7.58	7.81	4.54	4.74
		Maple Glen	7.75	7.60	4.89	4.87
	532C	AC Bravor	7.56	7.89	4.44	4.50
		Maple Glen	7.13	7.30	4.53	5.21
LSD_a			0.5	1.2	0.86	0.95
LSD_b			0.8	1.3	0.72	0.78

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TABLE 13 (cont'd)

The colony forming unit (log cfu gm dry root^{-1} and log cfu gm dry soil^{-1}) values for PGPR strains, in association with *B. japonicum* strains, and soybean cultivars from the fumigated field trial (experiment 1).

			(log cfu gm dry root^{-1})		(log cfu gm dry soil^{-1})	
PGPR	*B. japonicum*	Cultivar	June 20	Aug. 13	June 20	Aug. 13
PGPR			***	NS	***	NS
B. japonicum			***	NS	*	NS
cultivar			NS	NS	*	NS
PGPR **B.japonicum*			NS	NS	**	NS
PGPR*cultivar			NS	NS	**	NS
PGPR**B.japonicum**cultivar			**	NS	NS	NS

Means within the same column were analyzed by an ANOVA protected t test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot (PGPR strains) unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference and significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

At the nonfumigated site, the population density of both PGPR 2-68 and 1-102 on the root and in the soil had decreased by the second sampling, except in the case of PGPR 2-68 co-inoculated with *B. japonicum* onto soybean cultivar AC Bravor, where the population density remained very high both on the root and in the soil at the nonfumigated site (Table 12). Conversly, the population density of both PGPR 1-102 and 2-68 increased by the second sampling both on the roots and in the soil at the fumigated site.

Experiment 2

There was no difference in the number of cfu g^{-1} root at the first sampling between PGPR 2-68 and 1-102, howerver, at the second sampling PGPR 2-68 inoculated directly onto soybean cultivar AC Bravor had the highest population density (Table 14). There was no difference in the number of cfu g^{-1} soil between the first and second sampling for either PGPR (Table 14). There was not a great reduction in the population density of either PGPR associated with the root between the first and second sampling.

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TABLE 14

The colony forming unit (log cfu gm dry root^{-1} and log cfu gm dry soil^{-1}) values for PGPR strains and soybean cultivars from nonfumigated field trial (experiment 2).

PGPR	Cultivar	(log cfu gm dry root^{-1}) June 20	(log cfu gm dry root^{-1}) Aug. 13	(log cfu gm dry soil^{-1}) June 20	(log cfu gm dry soil^{-1}) Aug. 13
1-102	AC Bravor	5.66	5.32	3.99	2.98
	Maple Glen	5.81	5.54	3.68	3.78
2-68	ACBravor	6.67	6.68	4.12	3.08
	Maple Glen	6.23	5.66	4.24	3.37
LSD_a		0.97	0.83	0.29	0.99
LSD_b		1.00	0.91	0.28	0.98
PGPR		NS	*	*	NS
cultivar		NS	NS	NS	NS
PGPR*cultivar		NS	**	*	NS

Means within the same column were analyzed by an ANOVA protected t test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot (PGPR strains) unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference and differences at the 0.1, 0.05, and 0.01 levels, respectively.

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At the fumigated site, there were more cfu g^{-1} root for PGPR 2-68 than PGPR 1-102 at the first sampling, while there was no difference between the two PGPR at the second sampling. PGPR 2-68 inoculated onto AC Bravor plants had higher population densities in the soil (rhizosphere) than PGPR 2-68 inoculated onto Maple Glen plants. There was no difference between the two PGPR in terms of the number of cfu g^{-1} root or cfu g^{-1} soil at the second sampling at the fumigated site (Table 15). There were increases in the population densities for both PGPR associated with the root and in the soil (rhizosphere) by the second sampling (Table 15).

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TABLE 15

The colony forming unit (log cfu gm dry root^{-1} and log cfu gm dry soil^{-1}) values for PGPR strains and soybean cultivars from a fumigated field trial (experiment 2).

		(log cfu gm dry root^{-1})		(log cfu gm dry soil^{-1})	
PGPR	Cultivar	June 20	Aug. 13	June 20	Aug. 13
1-102	AC Bravor	5.74	6.3	3.69	3.67
	Maple Glen	5.30	6.7	2.97	4.44
2-68	ACBravor	7.26	7.8	4.31	4.57
	Maple Glen	6.91	7.3	2.98	4.38
LSD_a		0.89	0.7	0.74	2.1
LSD_b		0.81	1.9	0.93	1.5
PGPR		*** NS	*	NS	
cultivar		NS	NS	***	NS
PGPR*cultivar		NS	*	NS	NS

Means within the same column were analyzed by an ANOVA protected t test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot (PGPR strains) unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference and differences at the 0.1, 0.05, and 0.01 levels, respectively.

DISCUSSION

Plant growth-promoting rhizobacteria strains *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 were selected following previous controlled environment studies (Zhang et al., 1996b) in which nine PGPR strains were tested for effects on soybean plant growth, development, nodulation and nitrogen fixation over a range of RZTs under controlled environment conditions. *S. liquefaciens* 2-68 was shown to perform well at an optimal RZT (25°C), while *S. proteamaculans* 1-102 performed best at suboptimal RZTs ranging from 18 to 15°C.

Colonization of soybean plants varied among PGPR strains and soil conditions. In experiment 1, at the nonfumigated site, PGPR 2-68 colonized soybean plant roots more efficiently than PGPR 1-102 at the first sampling, while there was no difference by the second sampling which indicates that PGPR 2-68 was able to grow and colonize the root more effectively initially but over time PGPR 1-102 was able to grow and colonize the roots as effectively as PGPR 2-68. PGPR 2-68 was able to proliferate successfully in the soil (rhizosphere) as indicated by high cfu values at both samplings.

Another interesting observation is that the population densities of both PGPR 2-68 and 1-102 with the different combinations of *B. japonicum* and soybean cultivars had decreased in the second sampling as compared to the first, except for the combination of PGPR 2-68, *B. japonicum* strain USDA110 and soybean cultivar AC Bravor where the population density had increased in the second sampling compared to the first at the nonfumigated site. These observations indicate that PGPR 2-68 cells can survive and colonize the roots of the soybean plants effectively in the presence of other soil microflora elements, and that they can tolerate the change in field conditions, including temperature, over time without reductions in population density.

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At the fumigated site, where assumely, no other microflora compete with the PGPR, PGPR 2-68 showed the same pattern as in nonfumigated conditions. In addition, the population density of both PGPR had increased by the second sampling as compared to the first. These observations suggest that both PGPR were able to survive and increase in number in the absence of other microflora that would normally compete with them and reduce their population density.

The results of experiment 2, in the absence of *B. japonicum*, were different from those observed in experiment 1. There was no difference between the two PGPR in the number of cfu g^{-1} root at the first sampling, while at the second sampling the combination of PGPR 2-68, *B. japonicum* USDA110 and AC Bravor had the highest number of cfu g^{-1} root. There was no difference in the cfu g^{-1} soil between the two PGPR strains at both samplings of the nonfumigated site.

At the fumigated site, in experiment 2, the same pattern was seen as in experiment 1. PGPR 1-102 also had more cfu g^{-1} of both roots and rhizosphere soil. There was no difference in the number of cfu g^{-1} root or cfu g^{-1} soil between the two PGPR at the second sampling for both experiments.

Root colonization by introduced bacteria is considered as an important step in the interaction of beneficial bacteria with the host plant. A rapid growth rate was suggested to be an important characteristic for successful rhizosphere colonization (Rovira et al., 1983; Schorth et al., 1986). De Weger et al. (1987) suggested that non-motile mutants colonize the roots less effeciently than the corresponding wild types, while others found that non-motile mutants and the corresponding wild types do not differ in their colonizing ability (Parke et al., 1986; Scher et al., 1988). Chemotaxis of bacteria to exudates was reported (Reinhold et al., 1985; Scher et al., 1985), but the direct relationship between chemotaxis and successful colonization

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remains unclear. Movement along the root was also reported to be very important for a successful root colonization (Chao et al., 1986; Schippers et al., 1987). Adherence has also been suggested as an important feature for rhizospheere competence and survival (Schippers et al., 1987; Vesper, 1987). Cells of bacteria in the genus *Serratia* are motile (Prescott, 1993).

Fluorescent pseudomonads, isolated from the crop rhizosphere, are characterized as a highly rhizosphere competent as they are capable of root colonization. This accounts for their predominance among the PGPR. Several traits of the pseudomonads aid them in seed colonization, such as higher cell division and motility (Arora et al., 1983; Scher et al., 1985). However, these traits may not be directly relevant to subsequent root colonization. For example, Howie et al. (1987) found that three nonmotile mutants of *P. fluorescence* colonized wheat roots as effectively as their motile parents. Flourescent pseudomonads are able to establish high population densities in the rhizosphere (Bahme et al., 1987; Suslow, 1982), an important characteristic for the production of consistent plant growth responses (Bahme et al., 1987; Klein et al., 1990; Kloepper et al., 1980 and 1985; Kloepper et al., 1991; Parke, 1991; Suslow, 1982). In general, there has been little investigation of species in the genus *Serratia* as potential PGPR.

Studies on rhizosphere colonization have been reviewed recently by van Elsas et al. (1990), Kloepper et al. (1992) and Kluepfel (1993). Van Elsas et al., (1990) reported that lack of consistent effectiveness of the inoculant prevent successful application of PGPR strains in to the soil. This always related was to ineffective colonization of the plant, as well as poor survival and/or low activity of the introduced population. Xu et al. (1986) and Bull et al. (1991), demonstrated a positive relationship between root colonization by a PGPR strain and disease suppression, suggesting that methodologies which improve root colonization may also improve the

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performance of a PGPR strain in soil. The extent and amount of root colonization needed by a PGPR strain to increase plant growth rely on numerous interrelated factors. The choice of methods used to try to increase rhizosphere colonization and plant growth has to take these factors into consideration (Stephens, 1994).

Hebbar et al. (1992) reported that the colonization and spread of *Pseudomonas cepacia* (which acts as a bio-control agent against *Fusarium moniliforme*) on the roots and in the rhizosphere of maize depends on the amount of inoculum on the seed. However, this was not a universal observation. For example, the colonization of introduced pseudomonad strains on maize (Scher et al., 1984) and wheat (Juhnkle et al., 1989) was shown to be independent of the initial inoculum level. It is obvious that under certain conditions, increasing the level of inoculum could increase the rhizosphere competence of some, but not all, bacteria.

In a previous study, we found that co-inoculation with PGPR and *B. japonicum* improved plant growth, development, yield components, and final grain and protein yield under field conditions at both fumigated and nonfumigated sites. Also application of PGPR with the *B. japonicum* directly onto the seeds in the furrow at the time of planting also improved plant growth at the fumigated site. The effects of PGPR *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 on plant growth, development, and final protein yield were shown to be not different, which was attributed to variations in field soil temperature during the entire soybean growing season. In addition, a recent study at McGill university found that co-inoculation of PGPR and *B. japonicum* accelerated the processes of soybean nodulation and the onset of nitrogen fixation under short season conditions.

Zhang et al., (1996b), in a controlled environment experiment, showed that both *S. liquefaciens* 2-68 and *S. proteamaculans*

1-102 stimulate plant growth, development, and plant photosynthesis. At an optimal RZT (25°C) *S. liquefaciens* 2-68 was reported to increase plant leaf development and dry matter accumulation, while at 15°C RZT *S. proteamaculans* 1-102 increased plant dry matter accumulation.

In summary, the results of this study indicated that PGPR 2-68 was able to grow and survive better than PGPR 1-102 under short season conditions. A previous work, has shown that the combination of PGPR 2-68 with AC Bravor plants had increased leaf area, seed number, and grain protein yield suggesting that there is a relationship between the ability of these PGPR to colonize the roots of the soybean plants and their ability to stimulate soybean nodulation, nitrogen fixation, plant growth and physiological activities under short season conditions.

EXAMPLE 4

Effect of PGPR and Kinetin on plant growth development, nodulation and yield of soybean grown under short season conditions

In order to test whether the effect of PGPR could be further modulated by the addition of additional factors, field experiments were performed. Briefly, the experiment was conducted in 1997 and involved two soybean cultivars (Maple Glen and Bayfield), three kinetin concentrations (0, 0.1 and 5 µm), and in the absence or presence of PGPR strains (1-102 or 2-68). Soybean plants were harvested to determine plant growth development and final grain yield at one month after emergence, the early podding stage, and final harvest. The results of this study are summarized below as follows:

One month after emergence

The application of 5 µm kinetin increased total aboveground dry matter biomass per soybean plant by 12.5% when

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compared to the control (P=0.05). There was no difference in dry mater biomass between the two kinetin concentrations (Figure 1).

Compared to 0.1 µm kinetin, the application of 5 µm kinetin produced 54 and 83% more nodules and nodule weight per plant; and 66 and 87% more nodules and nodule weight per plant, respectively, were produced than the control (Figures 2 and 3). The PGPR strain 1-102 with the 5 µm kinetin treatment showed the highest nodule number per plant while it had the lowest nodule number at 0.1 µ m kinetin (P< 0.06) (Figure 3).

Early podding stage

The application of 0.5 µm kinetin resulted in 47 and 62% more nodules per plant than the 0.1 µm kinetin and the control treatment, respectively. Nodule number was not different from the 0.1 µm kinetin and control treatment on either cultivar (Figure 4). The two soybean cultivars responded differently to the kinetin concentrations in terms of nodule weight and root weight per plant. This finding corroborates the well known findings that the genotype of the cultivar affects its response to external stimuli (i.e. nitrogen fertilizers and the like). Bayfield with 5 µM resulted in the highest nodule weight when compared to its with 0.1 µM. (Figure 5). Under the 5 µM kinetin concentration, Maple Glen had the lowest root weight per plant while Bayfield had the highest (Figure 6).

At final harvest the following were recorded

The application of kinetin resulted in 5.2 cm taller plants than the control treatment over the two cultivars (Figure 7). There was no difference in plant height between the two kinetin concentrations. Kinetin at 0.1 µm had the lowest 100 seed weight when compared to kinetin at 5µm and the control. One hundred seed weight was not differ from kinetin at 5 µm and the control (Figure 8). Bayfield was taller, had more total aboveground dry matter biomass per plant, more seeds and more pods per plant than Maple Glen, but had lower 100-seed weight than that of Maple Glen (Figures

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7 and 9). Bayfield with 5 µm kinetin resulted in the highest seed number per plant while Maple Glen had the lowest ($P<0.07$) (Figure 10). PGPR 1-102 with 5 µm kinetin showed the highest grain yield while the lowest was without kinetin application and the same PGPR strain (Figure 11). However, the grain yield was not different from the combination of PGPR 102 and the 5 µm kinetin treatment and the control (no PGPR and kinetin addition).

In summary, one month after emergence, soybean plant growth, nodule formation and development increased with the application of the highest kinetin concentration; the combination PGPR strain 1-102 with 0.5 µm resulted in a positive response for nodule development. At the early podding stage, the application of 0.5 µm kinetin promoted nodule formation but not plant growth; Bayfield with 5 µm kinetin produced the highest root weight. At final harvest, the application of kinetin resulted in taller plants; Bayfield with 5 µm kinetin resulted in the greatest number of seeds per plant while the combination of PGPR 1-102 with 5 µm kinetin resulted in more grain yield than the same PGPR without kinetin.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

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WHAT IS CLAIMED IS:

1. A composition for enhancing nodulation of a legume grown under environmental conditions that inhibit or delay nodulation thereof, the composition comprising an agriculturally effective amount of a PGPR strain with a suitable carrier medium.

2. The composition of claim 1, wherein said PGPR strain is of the genus *Serratia*.

3. The composition of claim 2, wherein said PGPR strain is chosen from the group selected from *Serratia proteamaculans* and *Serratia liquefaciens*.

4. The composition of claim 3, wherein said *Serratia proteamaculans* strain is PGPR strain 1-102 and said *Serratia liquefaciens* strain is PGPR strain 2-68.

5. The composition of claim 1, wherein said environmental condition that inhibits or delay nodulation is low root zone temperature.

6. The composition of claim 1, wherein said agriculturally effective amount of a PGPR strain is from about 10^4 cells to about 10^{10} per ml.

7. The composition of claim 6, wherein said agriculturally effective amount of said PGPR strain is from about 10^6 to about 10^8 cells per ml.

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8. A method for enhancing nodulation of a legume grown under environmental conditions that inhibit or delay nodulation thereof, comprising:

a) inoculating in the vicinity of one of a seed and root of said legume with a composition comprising an agriculturally effective amount of a PGPR strain with a suitable carrier medium.

9. A composition for enhancing grain yield and protein yield of a legume grown under environmental conditions that inhibit or delay nodulation thereof, the composition comprising an agriculturally effective amount of a PGPR strain with a suitable carrier medium.

10. The composition of claim 9, wherein said PGPR strain is of the genus *Serratia*.

11. The composition of claim 10, wherein said PGPR strain is chosen from the group selected from *Serratia proteamaculans* and *Serratia liquefaciens*.

12. The composition of claim 11, wherein said environmental condition that inhibits or delays nodulation is low root zone temperature.

13. The composition of claim 1, further comprising a cytokinin.

14. The composition of claim 9, further comprising a cytokinin.

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15. A method for enhancing grain yield and protein yield of a legume grown under environmental conditions that inhibit or delay nodulation thereof, comprising:

a) inoculating in the vicinity of one of a seed and root of said legume with a composition comprising an agriculturally effective amount of a PGPR strain with a suitable carrier medium.

16. The composition of claim 1 comprising more than one PGPR strain.

17. A composition for increasing nodule formation of a legume grown under environmental conditions comprising an agriculturally effective amount of a cytokinin and of a PGPR strain.

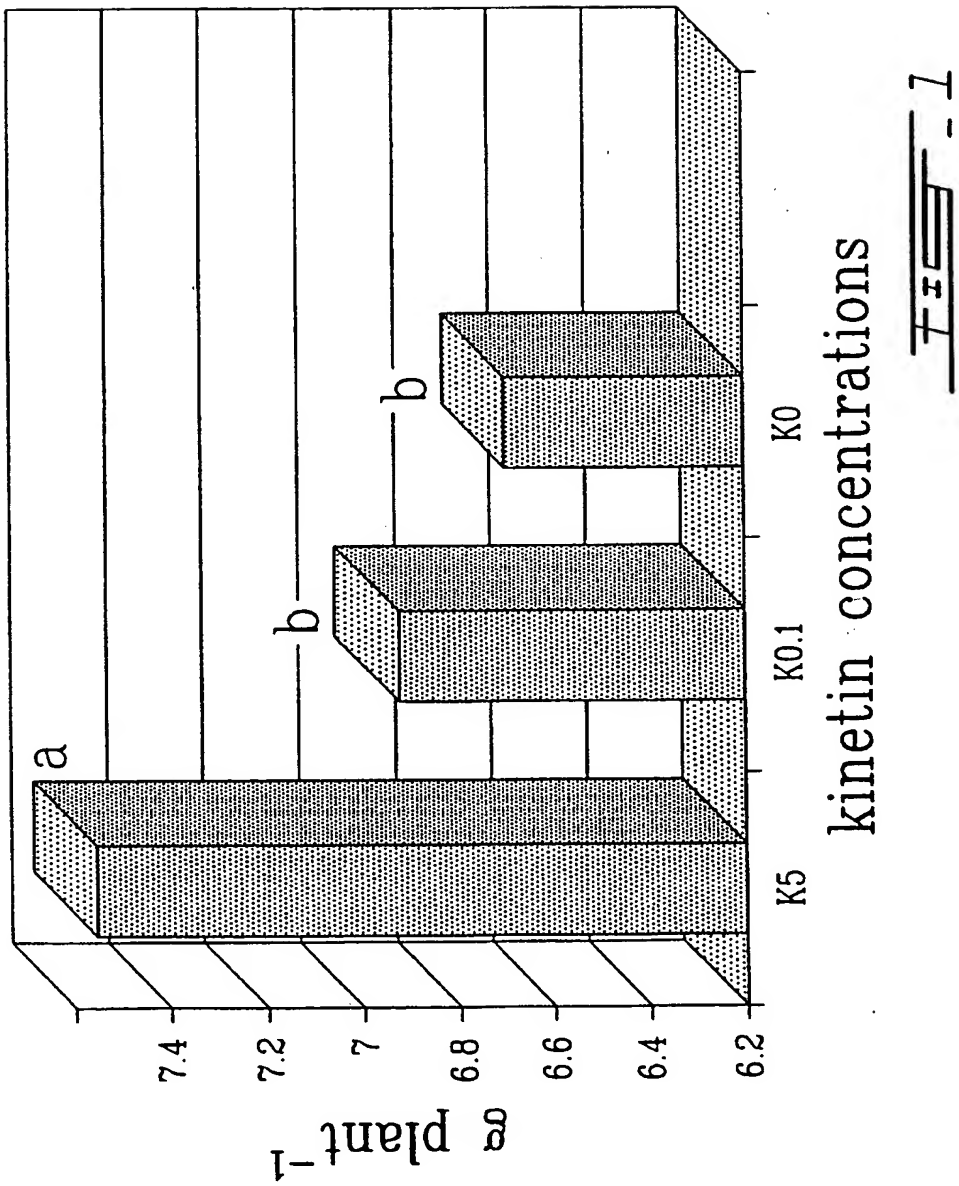

Fig - 1

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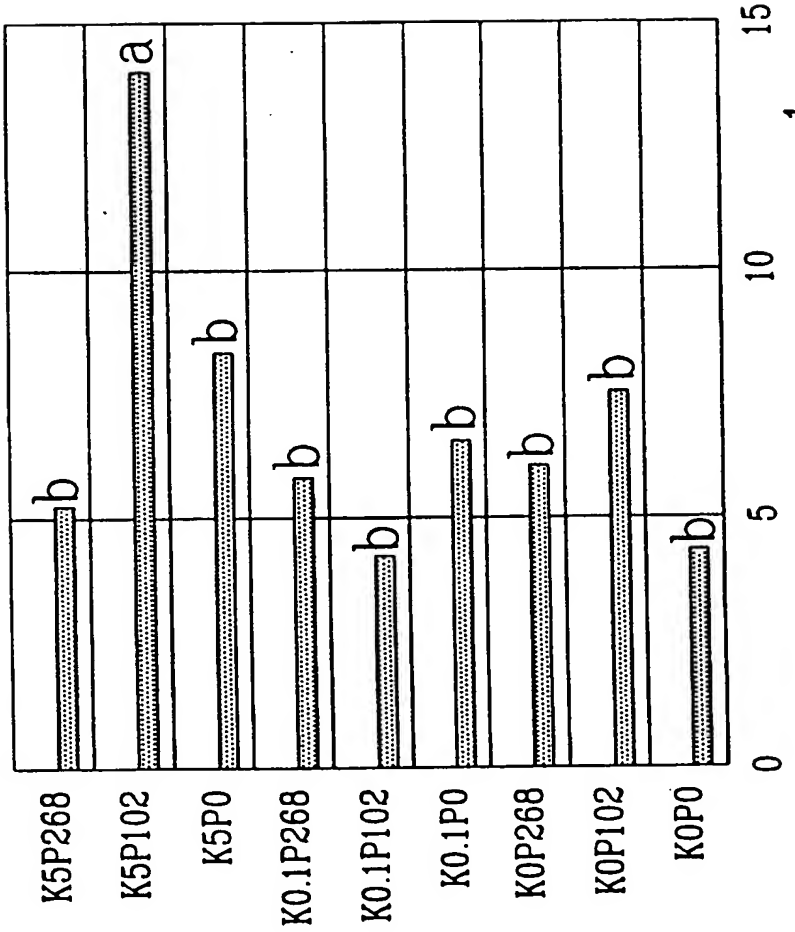

Fig - 7

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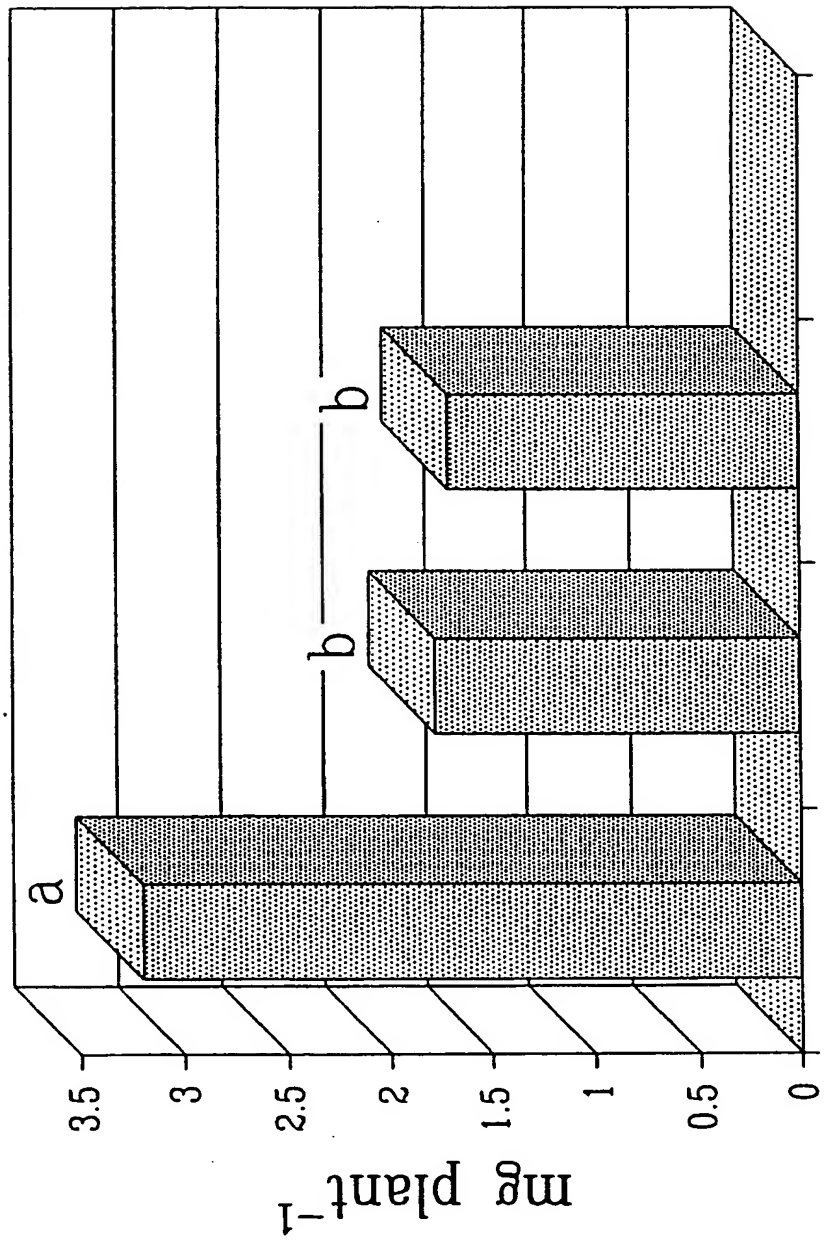

Fig. 3

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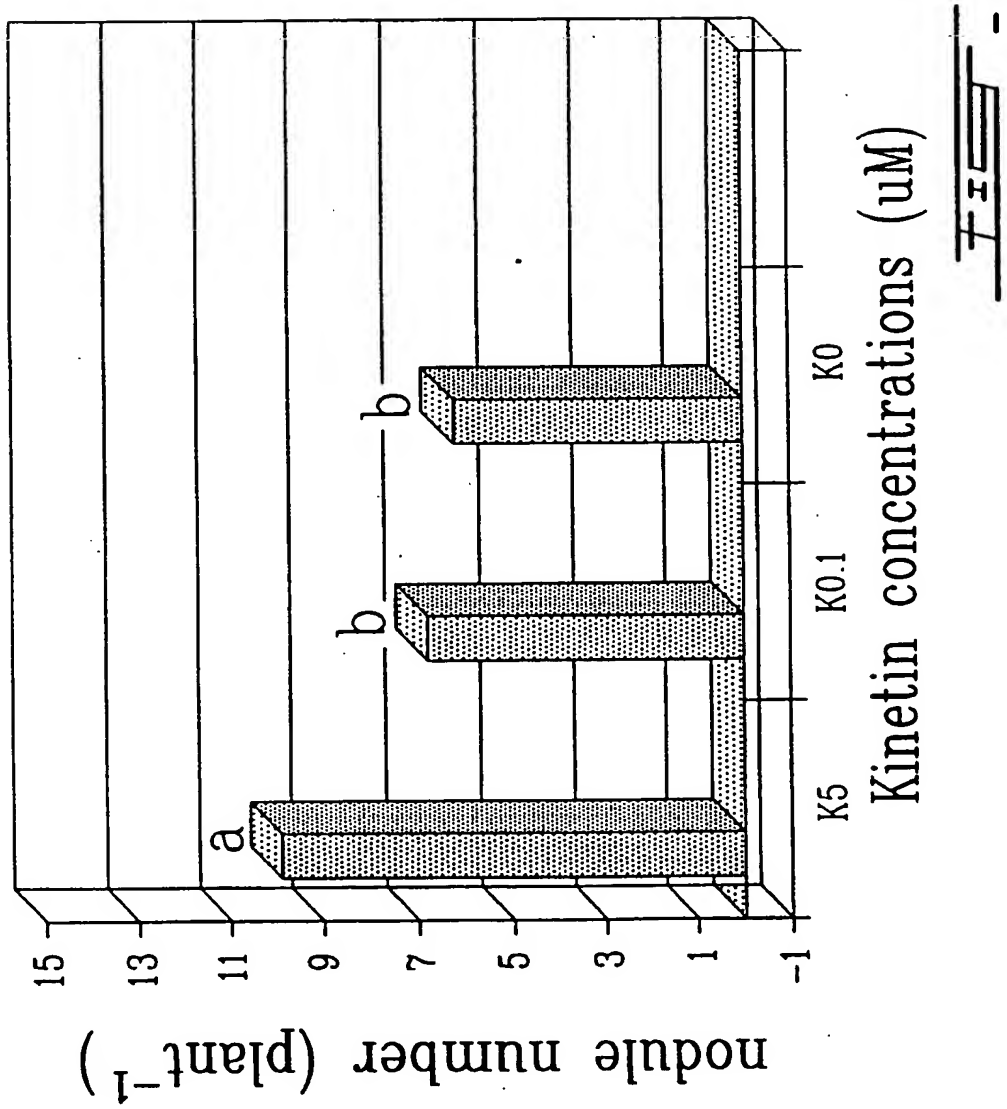

Fig - 4

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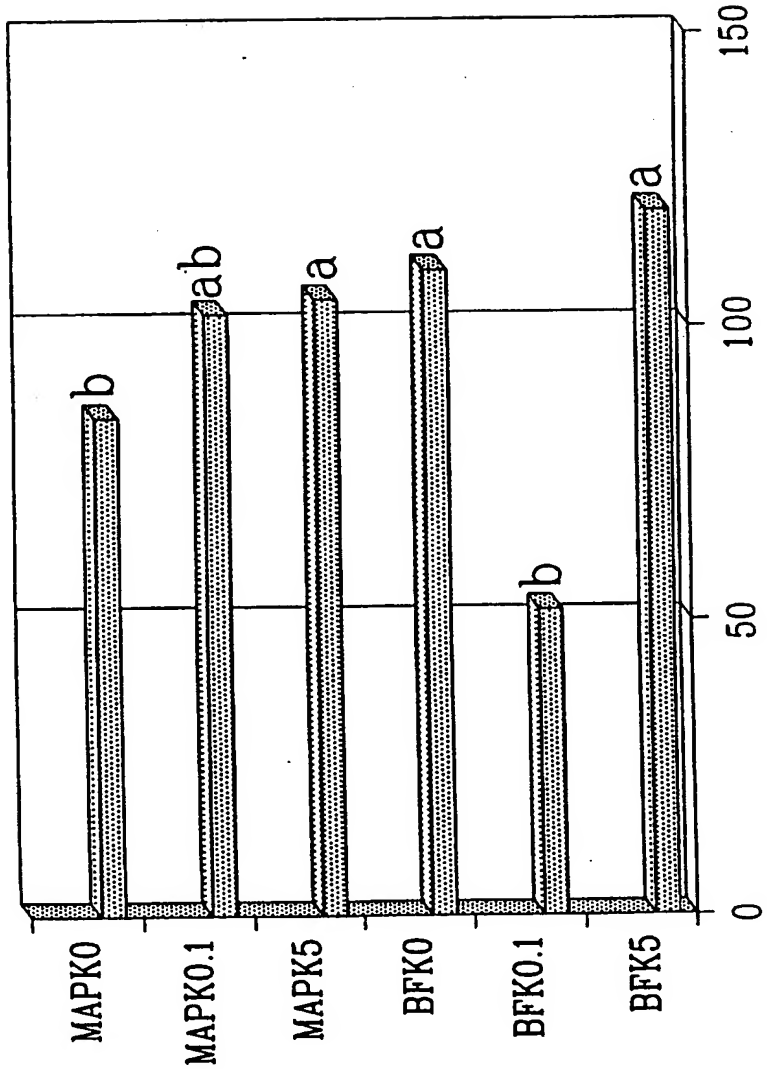

Fig. 5

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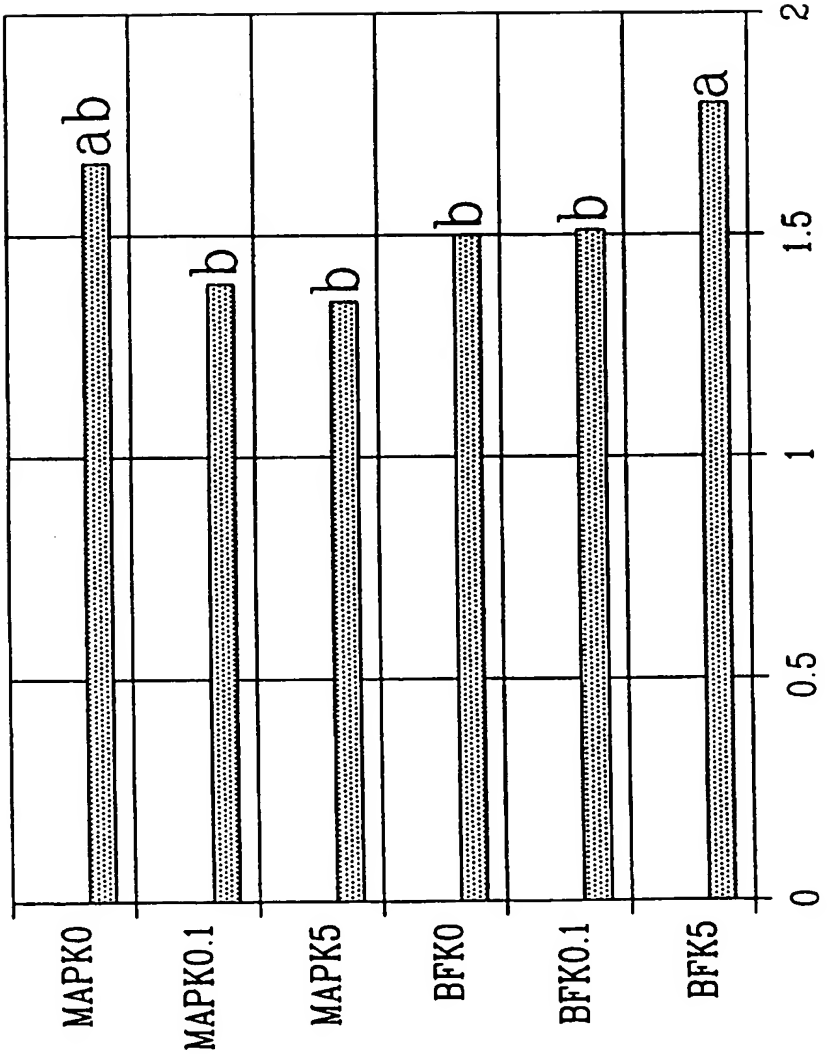

Fig - 6

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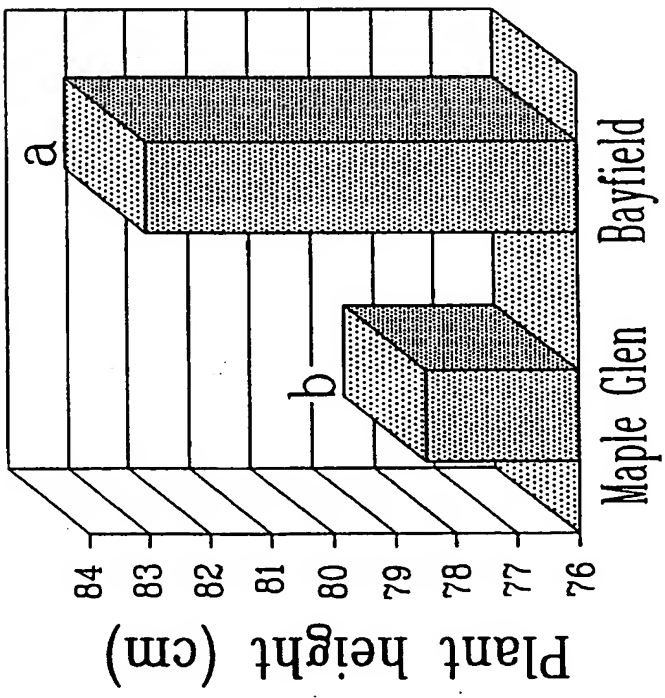

Fig - 7A

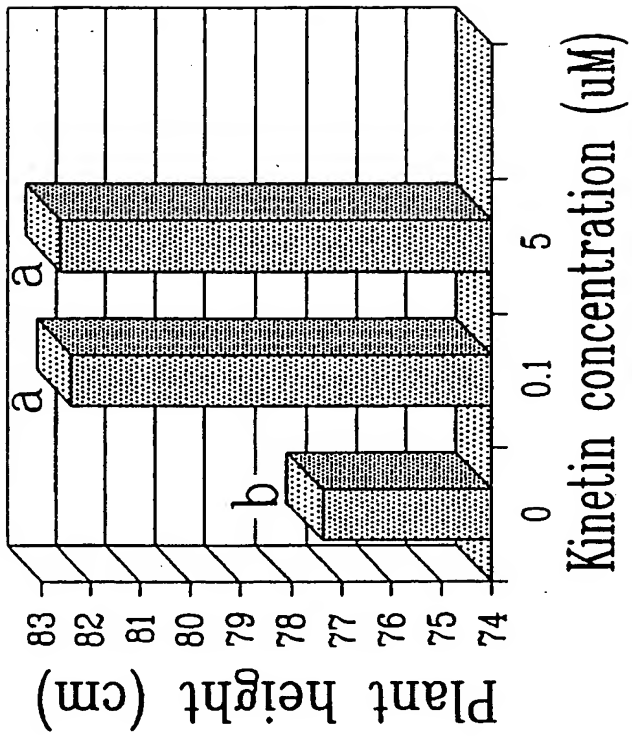

Fig - 7B

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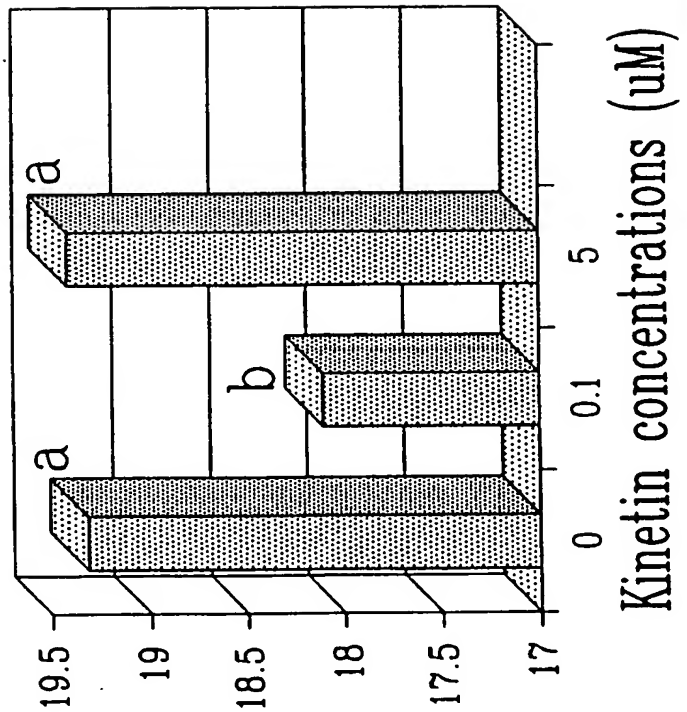

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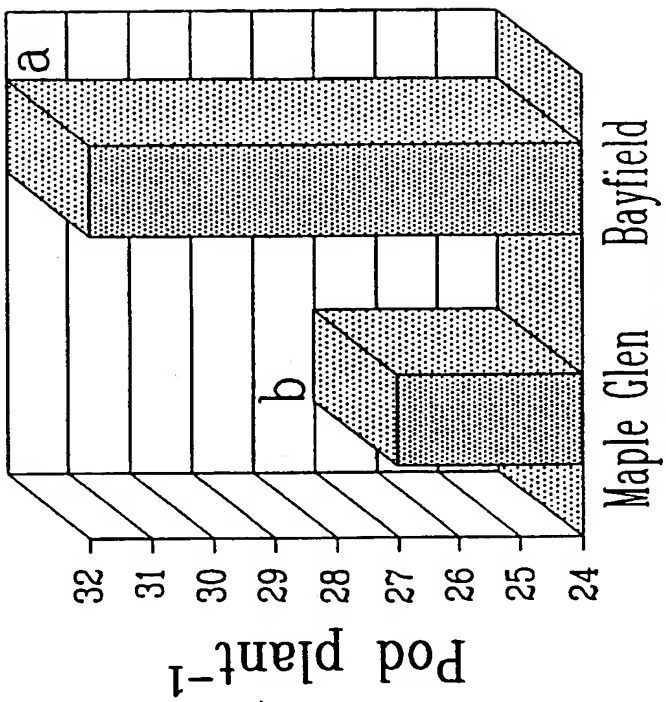

Fig - 9B

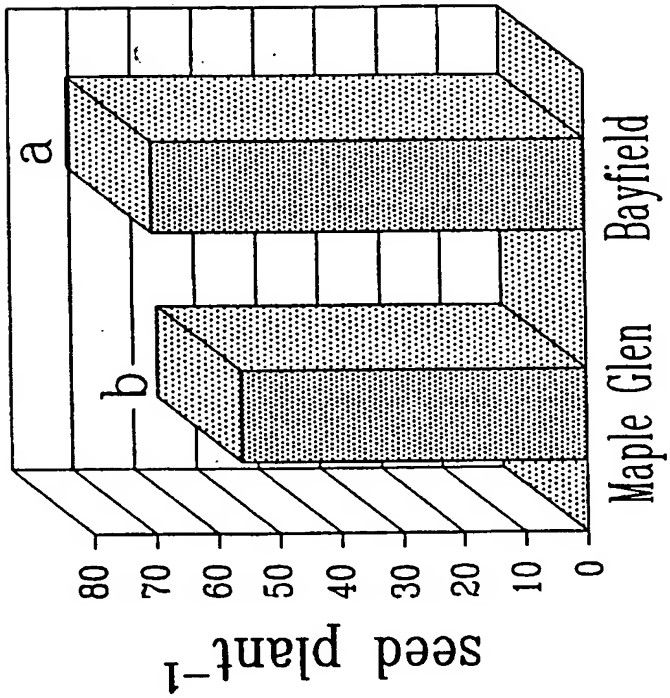

Fig - 9A

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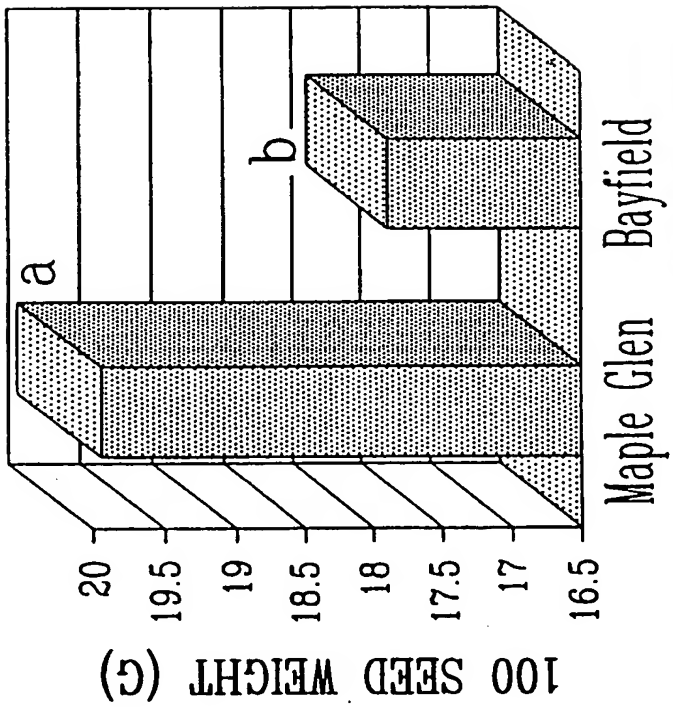

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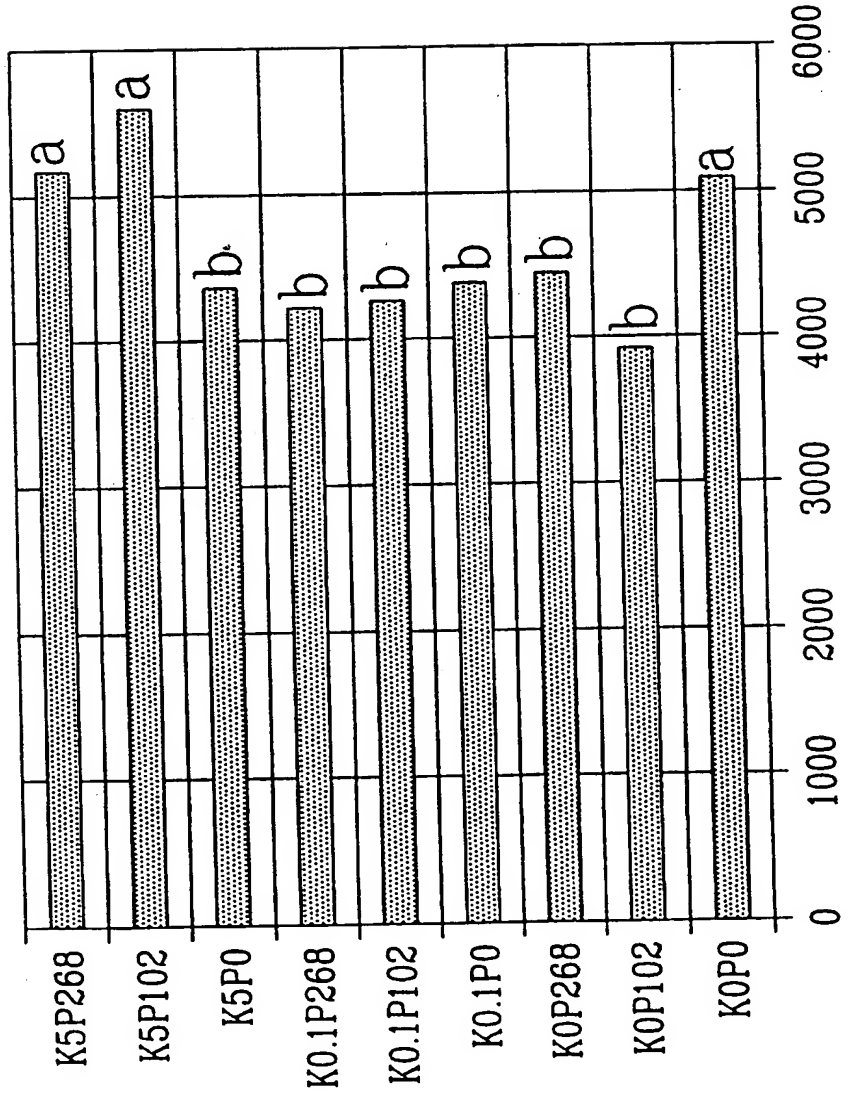

Fig - 11

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